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(57) Abstract

A method for detecting the presence of wheat having a D-genome in for example a processed food product is described. The method comprises extracting DNA from said product and detecting the presence of a nucleotide sequence which is characteristic of the D-genome and in particular is derived from the Dgas44 sequence or from the PSR128 intron. The sequence is suitably detected using PCR. A particular sequence within each of the Dgas44 and the single copy PSR128 intron has been identified as being a suitable target for detection. The method is useful in detecting contamination of flour, semolina, or pasta products which are made from *T. durum* by bread wheat. Reagents useful in the method as well as kits for conducting the method are also described and claimed.

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DETECTION OF D GENOME IN WHEAT SPECIES

The present invention relates to a method for detecting certain species of wheat such as common wheat (*Triticum aestivum*), in particular when these are present in food products such as pasta, as well as nucleic acids which are useful in said method.

Historically wheat is thought to have arisen as the product of a 'fortuitous' cross that is believed to have occurred about 10,000 years ago between a wild goat grass (*Aegilops sp*) and the ancient wild species of einkorn (*T. urartu*). The new species (emmer wheat, *T. dicoccoides*) having two sets of 7 chromosomes i.e. 28 in total. This spontaneous and rare doubling of the chromosomes gave the new species the distinct advantage that instead of being a 'sterile' hybrid it was able to produce the normal pollen and egg cells required for seed production. It was this emmer wheat that essentially formed the basis of early agriculture. The cultivated emmer wheats appear to have been subjected, whether intentionally or not to selective pressures that discriminated in favour of the wheat that had more durable seed heads and stayed on the stem during harvesting and were easier to thresh. During the subsequent years a range of emmer-related wheats emerged and one of the developments was that of *T. durum*, a wheat that produced a rough and particular flour (semolina) that was essentially devoid of fine flour particles. This wheat was grown particularly in countries adjacent to the Mediterranean and also in areas of America and has found particular use in the production of pasta and related products.

In addition to the developments that happened to the *T. durum* wheat during this period a significant development also occurred to the emmer-related wheats that were spreading throughout Europe. This progression also involved a further chromosomal transfer and lead ultimately to the emergence of the bread wheats (*T. aestivum*). This process once more arose by the process of hybridisation, most probably between a cultivated emmer wheat and a wild goat grass. The net result of the hybridisation was to introduce a further set of 7 chromosomes, which once more doubled to produce a plant with a total of 42

chromosomes. It is reasonably certain that the goat grass that was the donor of the chromosomes was *A. squarrosa* (called *T. tauschii* in North America). These 7 chromosomes are collectively known as the 'D' genome and represent a major advance in terms of wheat development with particular regard to its bread making quality and the technological properties of the flour. These 7 chromosomes carry the alleles that encode for a group of proteins known as the glutenins. It is these proteins that produce the viscoelastic properties typically associated with bread dough, they therefore can have a major effect on the resulting bread quality. They are however regarded as reducing the quality of pasta product.

The majority of pasta products that are manufactured and offered for sale within the European community are made solely from Durum wheat (*Triticum durum* Desf) semolina and are considered to be superior products to those manufactured from common wheat (*Triticum aestivum* L), or mixtures of the two species of wheat. The area is controlled by legislation, particularly in Europe. For instance, Italian law currently prohibits the manufacture of pasta containing *T. aestivum* for sale in Italy but not for subsequent export. Several European countries including Italy, Spain and France take the firm view that the inclusion of common wheat in pasta is effectively adulteration. However, pasta made from the semolina of both wheat species is frequently manufactured for sale in Germany and Holland. Currently the United Kingdom does not have specific regulations regarding the composition of pasta. However, both the Food Safety Act 1990 and the Food Labelling Regulations 1984 make it clear that it is an offence to misdescribe a product or to offer it for sale in a misleading way, hence, the presence of common wheat in a pasta product must be declared on the product label.

Pasta manufactured from durum wheat which is intended for export outside the EU may contain a maximum of 3% common wheat to allow for adventitious cross contamination during the agricultural process. Such cross contamination is not unusual since there is a tendency to grow both types of wheat in similar environments and they are often grown in close proximity to one another.

It has been the case for many years that common wheat has traded at a substantially lower price than durum wheat and this has acted as an incentive to those adulterating pasta for financial gain.

As a direct result of these problems a number of analytical methods have been devised to check pasta products and to verify the label claims that are made, in order to enforce the various requirements of food legislation.

A number of different analytical methods are available to address the problem of detecting the presence of *T. aestivum* in *T. durum* pastas. Most rely upon detecting the products of the D-genome, a chromosome group that is specific to Hexaploid wheat and hence is absent from *T. durum*. These methods usually involve the extraction of water or alcohol soluble proteins (albumins or gliadins) prior to their separation by either isoelectric focusing (IEF) or by polyacrylamide gel electrophoresis (PAGE). The detection of albumins which are fractionated by IEF (P. Resmini, Tec Molitoria (1968) **19**, 145-150, P. Resmini et al., Tec. Molitoria **27**, 97-109) have for many years formed the basis of the official Italian test method.

Contamination can also be detected by PAGE of specific water extractable enzymes (polyphenol oxidases (Feillet, P and Kobrehel, K. (1972). Ann. Technol. Agric. **21**, 17-24, Feillet, P and Kobrehel, K. (1974). Cereal Chem. **51**, 203-209) and esterases (Cooke, R.J., Smith, T.M. and Ainsworth, C.C. (1986). Seed Sci Technol. **14**, 693- 704). Alcohol extractable low mobility oma-gliadins (Kobrehel, K., Agaga, P., Autran, J.C. (1985). Ann Fals Exp Chim. **78**, 109-117, Burgoon, A.C., Ikeda, H.S. and Tanner, S.N. (1985). Cereal Chem, **62 (1)**, 72-74.) which are encoded by the D-genome of non-durum wheats, when fractionated by PAGE have also been shown to be effective at detecting contamination.

Other methods include the analysis of a the secondary metabolite sitosteryl palmitate by either thin layer chromatography (TLC)(Gilles, K.A. and Youngs, V.L. (1964). Cereal Chem, **41**, 502-513, Berry, C.P., Youngs, V.L. and Gilles, K.A. (1968). Cereal Chem,

45 (6), 616-626) or more recently by reverse phase high performance chromatography (RP-HPLC) (Sarwar, M. and McDonald, C.E. (1993). Cereal Chem, 70, 405-411). The analysis of fatty acid sterol esters by TLC following petroleum ether extraction, being the current recommended method for the detection of *T. aestivum* adulteration of pasta within the EC.

Immmunochemical assays based upon either water soluble proteins (EP-A-540,432, Piazzi, S.E. et al., (1969). Cereal Chem, 46, 642-646, Piazzi, S.E. et al, (1972). Cereal Chem. 49 (1), 72-78) or alcohol(McCarthy, et al., (1992). Food Safety and Quality Assurance. (Morgan, M.R.A. and Smith, C.J. Williams, P.A., Eds), Elsevier, London, UK, 1991 (pub.1992), 411-416) soluble proteins have also been investigated. Investigations have been made using RP-HPLC, to detect and quantify the presence of *T. aestivum* contamination in *T. durum* semolina based on the fractionation and quantification of a group of γ/β gliadins (McCarthy, P.K., et al., (1990). J. Sci. Food Agric. 50. 211-226). This method has recently been extended to include the analysis of pasta products dried at low and elevated temperatures (Barnwell, P. et al.,(1994). J. Cereal Sci. 20, 245-252). Specific albumins present in *T. aestivum* have also been detected in semolina using RP-HPLC (de Bernardi, I, de Noni., and Pellegrino, L. (1994). Food Chem 51. 325-329).

All the of the methods listed above have their shortcomings. The principal limitation being that of protein denaturation occurring as a result of the drying processes during pasta manufacture. This denaturing effect of heat on the proteins assumes an increased importance due to the fact that pasta producers are tending to move to higher temperatures to effect quicker drying of the product. Such processing renders the enzymes inactive and hence the enzymic detection methods ineffective. Denaturation of many of the other proteins used as markers of *T. aestivum* contamination also occurs. Denaturation of the γ/β gliadins causes band broadening and a shift in elution time when subjected to HPLC, making accurate quantification of the contamination impossible. The alcohol soluble omega-gliadins however, due to their lack of cysteine residues are considerably more heat stable and survive moderate thermal processing. While these proteins survive as markers

of contamination, little is known about their subsequent quantification other than that their apparent recovery from high temperature processed pasta is decreased, making accurate quantification difficult. A further factor that adds to the difficulties encountered is the little studied effect of the environment on the expression of the proteins used as markers of contamination. It has been noted (Wrigley, C.W. and Shepherd, K.W. (1973). Ann. NY. Acad. Sci. **209**, 154-162) that the albumins and globulins used by Piazzi et al (supra.) can be affected by the environmental growth conditions of the wheat, raising questions concerning the plausibility of the results. While the effect of the environmental growth conditions on other groups of wheat proteins is little studied, there is evidence (Blumenthal, C.S., Barlow, E.W.R. and Wrigley, C.W. (1993). J. Cereal Sci. **18**, 3) that this factor may be more important than was previously thought. The consequential effect on quantitative studies is therefore unknown.

In addition, there are intrinsic difficulties in the detection limits achievable using these methods. The limit of detection of the *T. aestivum* specific omega-gliadins for instance, is about 2-3% and also that the accuracy of determination is about 2%. The International standard for durum wheat trading permits a total of 3% non-durum grains in a shipment of *T. durum* hence when considering if a durum pasta product is misdescribed all of these potential errors must be summed.

The problem is further compounded due to differential processing conditions used in pasta manufacture, the nature of which may not be known to the analyst. The precise effect of different thermal processing upon the analyses is at the present time unquantifiable, and so for any meaningful analysis, a set of 'control' pastas processed at standard conditions must be used to quantify the adulteration levels. However, this control would represent only a median position and would result in the under measurement of adulteration in pastas dried at higher temperatures. Conversely, adulteration in pastas dried using somewhat lower temperatures would be over estimated.

Due to the problems associated with the conventional methods of analysis. the applicants addressed the detection and quantification of contamination in for example *T. durum* pastas through the detection of DNA of the D-genome.

A charcteristic sequence which would give an indication of contamination has been designated "Dgas44" which is a sequence derived from *Ae. squarrosa*, which has been identified previously (McNeil. D, et al., (1994). Genome. 37, 320-327) and is shown hereinafter in Figure 1 (SEQ ID No 1). Plant DNA was isolated and this sequence was cloned and identified. It was thought to be highly repeated in the D-genome of hexaploid wheat. and appears to be virtually absent from the A and B genomes.

It was not clear however what the effect of the extensive processing of the wheat during for example pasta production, would have on the DNA. and whether it would be possible to identify any characteristic sequences in such a product.

The applicants have found however, that such a method is possible and provides an advantageous means of detecting contamination.

By detecting contaminant wheat at the DNA level. the problems associated with the denaturation of the proteins and also the perceived problem of the differential expression of particular proteins as a function of the environmental growth conditions are avoided.

The present invention provides a method for detecting the presence of wheat having a D-genome in a processed food product. which method comprises extracting DNA from said product and detecting the presence of a nucleotide sequence which is characteristic of the D-genome.

Suitable food products from which DNA may be extracted include milled wheat products such as semolina or flour. or more extensively processed products. in particular pasta including dried pasta products.

In a preferred embodiment, the nucleotide sequence which is detected comprises or is a D-genome specific derivative of the Dgas44 sequence.

In another preferred embodiment, the sequence detected is the PSR128 sequence of the D-genome shown hereinafter in Figure 9. Single copy sequences are particularly preferred where quantification of the amount of *T. aestivum* is required. When multiple copy sequences such as Dgas44 are used (~500 copies present in the average D-genome), it is not certain that the number of copies remains constant across all the varieties of *T. aestivum*. Unknown variation may impede accurate quantification. However, PSR128 includes a single copy sequence derived from the D-genome.

The said nucleotide sequence may be detected by a variety of methods which are well known in the art. They may for example be detected using Southern or dot blotting techniques, or more preferably, by a preliminary amplification of the target sequence, followed by detection of the amplified product. A particularly suitable amplification technique uses the polymerase chain reaction (PCR) after which the amplified product is suitably detected following electrophoresis on agarose gels.

McNeil et al (supra.) proposed three primers for use in the polymerase chain reaction (PCR) amplification of Dgas44. These are shown underlined in Figure 1. However, when these were used in an attempt to identify contaminant wheats in pasta samples, it was found that the bands were very feint and diffuse bands which made identification difficult and which could not be entirely overcome by varying the PCR conditions. The problems are thought to be due to the high copy number of this sequence in the genome, giving rise to a large amount of PCR products, not all of the same exact length. It is also possible that there is some degree of length polymorphism among genomic copies of this sequence. There have also been problems due to very faint PCR products in *T. uratu*, the durums and the negative controls, indicating that the primers are not completely specific for the D-genome.

The applicants have identified a particular region of the sequence which is highly specific to the D-genome and from where advantageous probes or diagnostic primers may be derived. This region comprises nucleotide 1732 to 2150 of the Dgas44 sequence and is shown hereinafter in Figure 2 (SEQ ID no. 2).

Thus in a preferred embodiment, the method of the invention involves the detection of all or part of the sequence of Figure 2, and more particularly, the sequence of Figure 3. Novel probes and primers which are useful is such a method form a further aspect of the invention.

Thus the invention further provides a nucleotide sequence which comprises or hybridises with all or part of the nucleotide sequence shown in Figure 2. Said sequence is suitably detected in the method of the invention.

Suitable parts of the sequences defined above are sufficiently long to allow specificity and identification. In general therefore, the parts will be at least 15 bases in length and preferably at least 20 bases in length.

In a particularly preferred embodiment, the nucleotide sequence comprises or hybridises with all or part of the nucleotide sequence shown in Figure 3 (SEQ ID No 3). This sequence corresponds to bases 1864 to 2150 of the Dgas44 sequence.

The expression "hybridises with" means that the nucleotide sequence will anneal to all or part of the sequence of Figure 2 under stringent hybridisation conditions, for example those illustrated in "Molecular Cloning, A Laboratory Manual" by Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

The length of the sequence used in any particular analytical technique will depend upon the nature of the technique, the degree of complementarity of the sequence, the nature of the sequence and particularly the GC content of the primer and the particular hybridisation conditions employed. Under high stringency, only sequences which are completely

complementary will bind but under low stringency conditions, sequences which are 60% homologous to the target sequence. more suitably 80% homologous, will bind.

Where the sequence is used as a labelled probe (for example a radiolabelled or fluorescently labelled probe for example using Southern or Dot blotting) for direct detection of the target D-genome sequence, the probe length will be quite long, for example of 100 bases or more.

In view of the relatively low levels of DNA which may be expected however, for example in contaminated pastas. an amplification technique such as PCR is suitably employed, and the amplified product detected. For use as a PCR primer, the nucleotide sequence of the invention is preferably from 10 to 30 nucleotides in length, suitably from 17 to 30 nucleotides in length. At least two such primers will be necessary in order to effect the PCR amplification as is well understood in the art. These will be non-complementary sequences which hybridise with the opposite strands of the target region of the genome when denatured and which flank the region to be amplified. Suitable primers for use in a nested PCR technique may also be derived for example from

Particularly preferred primers are as follows:

CTTCTGACGGGTCAGGGGCAC (SEQ ID NO 4)

CTGAATGCCCCTGCGGCTTAAG (SEQ ID NO 5)

GTCCTATATCTTGAGGCCGCAAG (SEQ ID NO 6)

AACCCACTGTACCTGAGTATATATC (SEQ ID NO 7)

SEQ ID Nos 4 and 6 are suitably forward primers for PCR amplification whilst SEQ ID Nos 5 and 7 are reverse primers.

A particularly preferred pair of primers comprise SEQ Id Nos 4 and 5.

In particular, said method comprises amplifying the sequence of Figure 2. or preferably of Figure 3, using at least two primers and detecting amplified product.

Preferably, the methods of the invention is carried out so that the amount of said species of wheat present in the sample is quantified. This may be done, simply by comparing signals produced for example using PCR amplification with those produced from control samples of known contamination levels. It has been found by carrying out PCR experiments using control pastas, that an increase in the amount of PCR product using various primers has been seen as the level of bread wheat contamination increases.

However, in practice, it may be necessary to carry out a quantification assay in pastas of unknown quality. In such cases, factors such as DNA concentration, DNA quality, etc. are likely to play a very large role in the observed amounts of amplification products. Although it may be possible to compare the PCR product from such a test sample to one from a control pasta, since DNAs from the test and control would, in all likelihood, have been made from pastas produced under differing conditions, in different locations etc. it is unlikely that the DNAs would be of the same quality, size etc. It has been found in fact that a direct link exists between the amount of DNA used as a target for amplification and the temperature at which the pasta was dried. The more elevated the drying temperature the more DNA is required to obtain the same level of specific product after amplification. This effect is probably attributable to the thermal degradation of the target DNA and the subsequent decrease in the number of DNA molecules able to act as a target for the amplification reaction.

Given these factors, and the sensitivity of the required quantification assay it is necessary to adopt a more rigorous method for the quantification assay.

In a preferred embodiment, an internal standard or reference product, not arising from the D-genome that can be used to calibrate the PCR-assays internally, is used. In one

such method, a second nucleotide sequence is also amplified by multiplexing with additional appropriate primers. This second sequence is one which is found on the A and B and D genomes. In this way, detection of the second nucleotide sequence will provide an internal standard, against which levels of the D-genome specific sequence can be assessed to give relative proportions of the components.

The skilled person, would be able to check to see if any particular sequence from a plant genome fulfilled this requirement using routine methodology. In fact, the applicants have found that suitable non-specific sequences may also be derived from the Dgas44 sequence. A particular example of such a sequence is that defined by bases 5 to 876 of the Dgas44 sequence as shown in Figure 1.

Alternatively, quantification is effected by amplifying said target sequence by the using a pair of primers in the presence of a known concentration of a further sequence which competes with the said sequence for a primer, but which is distinguishable from said sequence during detection, and comparing signals produced by said further sequence and said target sequence. This method is based upon a known protocol (Förster, E.(1994). Biotechniques. 16, 20-24). A schematic diagram of the method used is shown in Figure 4 hereinafter. Basically the method entails the generation of a "synthetic" PCR product, of slightly shorter length than the PCR product generated from the template DNA, but having the same primer sites at its termini. This short-product is engineered by a two-stage PCR process, using the original full-length PCR product as template. The method employs "chimeric" primers at one end of the PCR reaction, in our case the 3' terminus. This "short product" is then added to PCR reactions in differing concentrations, in which it can compete against the template DNA for the primers. The signal strength arising from a known amount of the short product is then used as a control to estimate the amount or number of copies of the original template, for example, the amount of contaminating Dgas44 sequence arising from D-genome contamination of durum wheat samples or pasta products.

This assay would be independent of the DNA concentration or level of DNA quality or average size. The applicants have generated a PCR product using primers of SEQ ID Nos 4 and 5. and a shortened version thereof ("short PCR product")as illustrated hereinafter, and have used this in a quantification assay.

Preferably, cloned short-PCR product in circular form, most preferably plasmid borne, is used. Such a product will be easy to generate in large quantities and may minimise 'contamination' of PCR procedures (such a sequence may be extremely "invasive" sequence, and extreme care has to be taken to ensure that this sequence does not contaminate other PCR reactions using closely related primers).

In addition, when using a PCR amplification technique, quantification is suitably effected using a fluorescence based PCR product quantification system such as the TAQMAN ™ from Perkin-Elmer. This system uses selective cleavage of a probe oligonucleotide during the PCR in order to provide a "real-time" picture of the progress of the PCR reaction. This system offers the potential for accurate quantification of PCR products. In essence, the amplification reaction is effected in the presence of a probe which includes a quencher moiety and a fluorescence reporter, said probe being arranged to be selectively cleaved during the amplification reaction so as to produce a flurorescent signal. The signal, which is generated by laser induction, is monitored by and the rate of increase used to calculate the amount of target sequence present in the sample. When referred to hereinafter, the probes will be referred to only by reference to the characteristic sequence which they contain. It should be understood that the fluorescent moiety and the quencher moiety are included.

Such reactions can be used to quantify the amount of *T. aestivum* present in a sample of known wheat content. However, since there are limitations on the accuracy of most methods of determination of the total wheat content, it may suitably form part of a comparative study, in which a PCR reaction and a TAQMAN™ probe designed to amplify and detect a sequence which is common to all the genomes is carried out in parallel. Such a reaction, in effect measures the amount of wheat DNA in the sample in

the reaction. The signal generated by the D-genome specific amplification reaction could then be standardised against this measurement, and so an accurate percentage figure for any contaminant *T. aestivum* can be established. The standardising reaction may be effected in a separate tube containing an equal aliquot of DNA being investigated. Alternatively, use of distinguishable laser dye mixtures would allow the reactions to be effected in the same reaction tube.

A useful TAQMAN probe which has been successfully been used in quantitative PCR reaction to amplify the sequence of Figure 3 is a probe based upon base numbers 1988-2014 of the Dgas44 sequence. In other words, the TAQMAN™ probe comprises SEQ ID No .10

$^{5'}$TTG GGA GGC ATG GTG AAA GTT GGT GAT$^{3'}$ (SEQ ID NO 10)

However, as mentioned above, quantification is more accurately carried out using a single copy sequence such as that found in the D-genome specific region of PSR128. PSR128 is an intron sequence derived from a cDNA clone. Techniques for mapping this probe are described by D.X. Xie et al., Theor. Appl. Genet (1993) 87: 70-74. The size of the intron differs in each of the genomes of hexaploid wheat. Figure 9 shows the PSR128 sequence found in the D genome (SEQ ID NO. 19). The D-genome copy of the intron includes a unique sequence (bases 62-114). By utilising this unique sequence, for example using PCR methods, the single copy PSR 128 sequence of the D-genome can be detected which can then be be most readily and accurately quantified.

Primers which will achieve this would be determinable by the skilled person. In general, at least one of the primers is derived wholly or partly from the D-genome unique sequence. Where the TAQMAN™ system is used, the probe may itself be derived from said sequence.

Examples of suitable primers for the amplification of the D-genome PSR 128 sequence are

$^{5'}$ATG GCT GGC TTC TAT TTC ATG$^{3'}$ (SEQ ID No. 12)

as the forward primer and

$^{5'}$CAC CTA CTC CTC CAC ACT TTG$^{3'}$ (SEQ ID No. 13)

as reverse primer.

The corresponding sites on the PSR128 D-genome sequence are underlined in Figure 9 hereinafter.

Alternative primers focus more closely on amplification of a smaller amplicon within the D-genome specific region. For example, a suitable pair of primers would be

$^{5'}$AAG GAG CTC GCC AAC GG$^{3'}$ (SEQ ID No 14)

as forward primer and

$^{5'}$AAC CGA GGG TCC AGA AGA GAC G$^{3'}$ (SEQ ID No 15)

as reverse primer.

These primers produce a small 116 base pair amplicon. Furthermore, a TAQMAN™ probe may further be employed to quanitate the PCR reaction, for example as follows:

$^{5'}$GCT AGA TGG CTG GCT TCT ATT TCA TGA TCC A$^{3'}$ (SEQ ID No 16)

The position of these primers and probes is indicated by underlining in Figure 10 hereinafter. The use of a smaller amplicon is preferable in the present case, both for kinetic reasons associated with the PCR reaction and as a result of the degradation which may have occurred during food processing, for example of pasta.

As illustrated hereinafter, a number of "control" pastas were made using precise levels of common wheat *T. aestivum* adulteration The range of adulteration levels covered were those causing most concern both to industry and the regulatory bodies. These were manufactured using three different drying regimes. These were 56°C, 80°C and 104°C representing the range of drying protocols widely used by industry.

It was surprisingly found that DNA could be isolated from these and other commercially available pastas through the use of conventional extraction procedures, for example using an extraction method based upon a phenol/chloroform mixture. The isolated DNA was detectable using the methods of the invention, and in particular, after suitable purification, it is suitable for both amplification using PCR and for use in hybridisation protocols.

The target DNA is preferably subjected to analysis as soon as possible since stored material may suffer from some degradation, possibly as a result of the co-purification of nucleases which may further degrade the template. When storage is necessary, it is preferable is nucleases are removed from the DNA, for example using a solid-phase cleanup procedure as is known in the art.

Using the methods of the invention. It has been found that very low levels of *T. aestivum* can be detected in pasta (as low as 0.1%) and that appropriate controls are required if accurate quantification is to be carried out.

In order to check the veracity of the results, a large number of commercial cultivars of both *T. durum* and *T. aestivum* have been assembled from around the world and have been investigated using the PCR assay and the D-genome specific Dgas44 primers. In addition to these cultivars, a large number of related species and amphiploid lines of wheat have also been assessed. The results showed that all of the *T. durum* cultivars gave negative results in respect of the amplification of the D-genome specific Dgas44 product. All *T. aestivum* cultivars produced the specific PCR product. The results suggest that the detection of the D-genome by the Dgas44 primers is highly unlikely to

be compromised by cross-reactions with other unrelated genomes. This finding was underlined by the use of other *Triticum* species and amphiploid (*Tritordeum*) lines.

Therefore this technique may be employed to identify the presence of D-genome characterising nucleotide sequences from DNA from any source, and therefore may be used for instance in the identification of particular species. Specifically the invention further provides a method for identifying the presence of a D-genome in a crop sample, which method comprises extracting DNA from said sample, and detecting the presence of a nucleotide sequence which comprises or hybridises with all or part of the nucleotide sequence shown in Figure 2, for example, by amplifying said sequence using PCR.

The skilled person would understand that changes can be made to, for example, the precise PCR conditions illustrated herein and still produce acceptable results. Particular aspects which may be varied include the use of different PCR reagents, number of PCR cycles, buffer conditions such as magnesium ion concentration, primer concentration, enzymes and enzyme concentrations, and thermal cycling apparatus. In general however, the PCR reactions will be effected with at least 25-30 cycles in the presence of a magnesium ion concentration of from 0.5 to 4.0mM, preferably about 2.0mM. Suitably, the polymerase enzyme used is a thermostable polymerase such as Taq polymerase. It is suitably present in concentrations of from 0.25U to 0.5U/50µl.

The invention will now be particularly described by way of example with reference to the accompanying drawings in which:

Figure 1 shows the nucleotide sequence of the Dgas44 sequence;

Figure 2 shows the region from base no. 1532-2150 of the Dgas44 sequence;

Figure 3 shows the region from base no 1864-2150 of the Dgas44 sequence;

Figure 4 illustrates the method used for synthesis of a PCR competitor template;

Figure 5 shows the results of a 25µl touchdown PCR 68-60 reaction using primers of SEQ 4 and 5, in which 10µl PCR product was run on a 1% agarose gel;

Figure 6 shows the results of a 25µl touchdown PCR 68-60 reaction using primers of SEQ ID Nos 4 and 5 and also SEQ ID Nos 8 and 9, and also primers in which 10µl PCR product was run on a 1% agarose gel;

Figure 7 shows the results of a dot blot with different amount of 56 pasta DNA per well, which was hybridised to a radiolabelled probe derived from Dgas44 sequence;

Figure 8 shows the results of a touchdown 68-60 PCR reaction using primers derived from Dgas44 in a 50µl reaction using 0.5µl Taq per tube, and with 10µl PCR product run on a 1% agarose gel;

Figure 9 shows the sequence of an intron sequence designated PSR128 in the D genomes of *T. aestivum*;

Figure 10 shows a portion of the sequences of Figure 9 with PCR primer and TAQMAN™ probe underlined;

Figure 11 shows a typical result of quantification assay of dried durum pasta using the TAQMAN™ methodology for a pure durum sample (0% adulteration) and a sample containing 1% adulteration:- the method determines the cycle threshold value (Ct) which is the PCR cycle or part of a cycle when the detected fluorescence differs from the 0% standard by 6 standard deviations. By plotting the Ct value against the known adulteration level within the standards, a calibration curve may be plotted :

Figure 12 shows typical plots similar to those of Figure 11 but using dried pasta with 0%, 1% and 2% adulteration (each in triplicate);

Figure 13 shows typical plots similar to those of Figure 11 but using dried pasta with 0%, 1%, 2% and 3% adulteration (each in triplicate);

Figure 14 is the calibration plots for the three sets of standard pastas (56, 80 and 104°C dried) assessed using a TAQMAN™ probe to Dgas44;

Figure 15 presents the calibration plots derived from all of the data presented in Figure 14 which would be used to assay pastas whose thermal history was unknown;

Figure 16 shows the calibration plots for the three sets of standard pastas (56, 80 and 104°C dried) assessed using the TAQMAN™ probe to the single copy sequence PSR 128; and

Figure 17 shows an individual calibration curve for the 104°C dried pasta standards. assessed using the the TAQMAN™ probe to the single copy sequence PSR 128.

Example 1

The preparation of semolinas of known levels of adulteration from accredited samples of *T. durum* (cultivar, Néodur) and *T. aestivum* (cultivar, Soissons).

25kg of accredited grains of the *T. durum* cultivar Néodur and a small amount of accredited *T. aestivum* (cultivar, Soissons) was obtained. Soissons is currently a major breadmaking variety of wheat and as such could be expected to be found adulterating *T. durum* grists destined for pasta production. Analysis showed the sample of Néodur to contain 11.4% protein and 14.6% moisture. The sample of Soissons contained 10.4% protein and 11.0% moisture. Electrophoretic analysis (Acid-PAGE) of the samples of Néodur and Soissons showed that both varieties were 100% pure.

The accredited Néodur was adulterated with Soissons on a weight basis (as below) to produce 0, 1%, 2%, 3%, 5%, 10% levels of contamination are shown in Table 1.

TABLE 1

% Adulteration	Néodur (g) *(T. durum)*	Soissons (g) *(T. aestivum)*
0	5000	0
1	3960	40
2	4900	100
3	4850	150
5	4750	250
10	2250	250

Durum grain was taken from its natural moisture content of 14.6% to a final moisture of 18.5% in two stages (a) an initial step of taking it to 17% moisture; and (b) a final addition of water to bring the moisture content to 18.5%. These steps were carried out over 8 hours.

The grains were then milled using a Buhler (Switzerland) test mill which had been dismantled and thoroughly cleaned prior to use in order to remove all traces of any contaminating *T. aestivum* flour. Only the first half of the mill was used, that producing semolina. In preparation for milling the test samples a non-accredited (normal *T. durum* mill grist) was used to set the mill to the required settings i.e. that designed to remove the bran layer from the hard *T. durum* grains while yielding a semolina that was as representative as possible of the product from the larger mills. Once the desired settings

were achieved, the mill was stripped down and vacuum cleaned before the test millings proceeded.

Deliberately adulterated Néodur was milled in ascending order of contamination with a thorough mill stripdown and vacuum clean between each test sample.

The yields of semolina are shown below in Table 2:

TABLE 2

% adulteration	Weight of grain used (g)	Semolina yield (g)	weight of semolina available for pasta production (200g retained for experimentation)
0	5000	3758	3550
1	4000	2723	2523
2	5000	3654	3450
3	5000	3467	3250
5	5000	3588	3388
10	2500	1843	1643

The moisture content of the adulterated semolina was then determined. This was assessed using two semolina samples (0 and 10% *T. aestivum* content). Both samples were placed in a Newport 400 NMR analyser and the result obtained. The results were as shown in Table 3:-

TABLE 3

T. aestivum content (%)	moisture %
0	16.76
10	16.92

An average moisture content of 16.80% was used in all subsequent work.

The semolinas were then turned into pasta using a small scale pasta press for the production that was capable of being completely cleaned out between samples and which had a very low dead space (and hence wastage). Doughs produced commercially from semolina generally contain 32% water and this recipe was adopted for the subsequent work with water addition being calculated as follows:-

dry solids (g) = wt semolina (g) - (wt semolina (g) * 16.8/100)
water addition (ml) = (dry solids (g) /0.68) - wt semolina (g)

The relatively small amounts of semolina (300-1300g) that were used in the series of experiments allowed mixing to be achieved using a Kenwood Chef mixer. The semolina was placed in the bowl of the mixer and while the blade revolved on the slowest setting, the water (@ 50°C) was slowly dribbled into the bowl. Initially, only two thirds of the required water was added over a period of 2 minutes. The blade was then stopped and the bowl scraped down before the mixer was restarted and the remaining one third of the water added. Mixing was continued for a further minute before the mix was transferred to the mixing compartment of the pasta press where it was allowed to mix for a further 3 minutes before extrusion commenced.

Prior to the extrusion of the pasta dough, the pasta press was thoroughly cleaned by totally dismantling the dye and screw mechanism. All parts were soaked and cleaned as necessary with the mesh and dye being subjected to cleaning by pressurised jets of water in a commercial die wash. The screw and die, once clean, were kept at 50°C prior to

drying and assembly ready for the next experiment. This enabled the desired running temperature to be achieved more quickly (important for quality reasons).

The pasta produced by the above method was dried by one of three regimes, representing the current commercial practices within Europe. All drying profiles (with the exception of the 104°C step within the very high temperature profile) were performed in a Hedinair (Kingsbridge Road, Barking By-pass, Barking, Essex) programmable hot air oven with relative humidity control and humidification. The 104°C step within the very high temperature procedure was carried out using a calibrated Gallenkamp model OV-160 hot air oven. The three temperature profiles used were as follows:-

1.low temperature method (56°C)

a) 12 h, @56°C, ΔT 5°C, 76%RH.

moisture content (by NMR) at the end of the procedure was 10.4%

2.'Normal' high temperature method (80C)

a) 40min, @60°C, ΔT 7°C, 69% RH.
b) 2h, @80°C, ΔT 7°C, 74% RH.
c) 2.5h, @75°C, ΔT 5°C, 80% RH.
d) 8h, @56°C, ΔT 5°C, 81% RH.

Moisture content (by NMR) at the end of the procedure was 10.0%

3.Very high temperature drying method (104°C)

a) 40min. @60°C, ΔT 7°C, 69% RH.

b) 1H, @80°C, ΔT 7°C, 74% RH.

c) 1h, @88°C, ΔT 8.5°C, 71% RH.

d) 1.5h, @104°C, ~90% RH.

e) 1.5h, @75°C, ΔT 5°C, 80% RH.

f) 6h, @56°C, ΔT 4°C, 81% RH.

Moisture content (by NMR) at the end of the procedure was 10.25%

The constraints of ensuring complete integrity of the samples of pasta that were produced and organising the three different drying regimes dictated that each semolina was turned into pasta on three different days. The production at the end of each day being dried by a particular temperature profile. This eliminated cross-contamination and allowed each drying regime to take place overnight.

To eliminate cross-contamination during manufacture pastas were produced each day in an ascending order of *T. aestivum* contamination.

To assess the cleanliness and to check the levels of adulteration in the pastas produced as 'controls' for the extraction of DNA, all pastas were examined by the traditional method of analysis i.e. omega-gliadin analysis. The results of this analysis confirmed the initial purity of the Néodur and the proportional increase in omega-gliadin content as the level of adulteration increased although the detection limits of the technique (2-3% when assessed visually) were also clear.

Samples of each 'control' pasta (~10g) were stored in stoppered glass pharmaceutical bottles (125ml) fitted with a tamper evident cap to ensure sample integrity. Each bottle had a numbered label indicating both the level of adulteration of the pasta and the drying conditions of the pasta sample it contained. Individual bottles are tracked via the use of

a log book giving details of both the user and the date used. All samples were stored in a locked cold room, in the dark at 4°C.

Example 2

DNA extraction from samples and detection using PCR.

DNA was isolated from the control pastas produced in Example 1 and using the phenol/chloroform method of Sharpe et al. (1988). Theor. Appl. Genet. 75, 289-290. Each pasta(5g) was ground into flour by milling for approximately 20 min after light crushing with a hammer. The flour was transferred to a 50 ml polypropylene tube and buffer "S"(20ml) comprising 100mM Tris.Cl (pH 8.5), 100mM NaCl, 50 mM EDTA (pH 8.0) and 2% SDS, was added. The mixture was thoroughly dispersed by vortexing.

A 10mg/ml proteinase K stock solution (100µl) was added per tube (final concentration 0.05 mg/ml). The tubes were then incubated at 65°C for 1-2 h with the occasional inversion to mix the contents. A phenol/chloroform mixture (20ml), which had been prepared by mixing 1 part TEN buffer saturated phenol, pH 7.8 : 1 part chloroform :isoamyl alcohol(24:1) was added to each tube and mixed gently by inversion to form an emulsion. Centrifugation at 2000rpm for 20 min separated phases although the addition of phenol/chloroform mixture and subsequent centrifugation was repeated if necessary.

Propan-2-ol (0.6 vol) was added and mixed by inversion. DNA was recovered by centrifuging at 10,000 rpm at 4°C. The DNA was washed with cold 70% ethanol and air dried. A further wash with 100% ethanol was performed to speed drying. The DNA was suspended in 1x TE buffer (5ml) (composed of 10mM Tris (pH 8.0) and 1mM EDTA (pH 8.0)) and kept at 4°C . Total solution can take several days, however, this can be speeded by careful warming at 65°C for up to 2hrs with gentle inversion. RNase stock solution (10µl) (comprising 1mg/ml RNase A and 100U/ml RNase T1 which had been heated to 100°C, 10min to destroy DNase activity) was then added and the solution incubated at 37°C for 1h. The phenol/chloroform extraction was then repeated using

phenol/chloroform (5ml), followed by gentle mixing by inversion and centrifuge at 2000rpm for 15 min.

The product was extracted using an equal volume of chloroform, a step which was repeated if material at the phase interface is excessive.10% volume sodium acetate (3M) (final concentration 0.3M) was added with mixing, followed by 2 vols ethanol (100%). After centrifuging at 10.000rpm @ 4°C, and washing in cold 70% ethanol, the DNA product was air dried and redissolved in 1.8-2 ml TE buffer (depending upon the size of pellet). This solution was stored frozen at -20°C.

Aliquots of each stock DNA thus produced was taken and purified using a column Chromatography method (Wizard PCR Preps, Promega, Madison, WI, USA). The purified DNAs were diluted to 20ng/µl as a working solution. This process was repeated twice, the first occasion giving cleaner DNA and a greater recovery.

In addition, more DNA was made from durum and hexaploid wheat plants were extracted using the phenol/chloroform method described above. Plants sown in batches of about 30 individuals of each sample yielded enough leaf material after two and a half weeks to provide leaf for two DNA extractions (approx. 5g leaf tissue per DNA extraction). The varieties sown were the hexaploids Chinese Spring and Soissons, and the durums Néodur and Primadur. Two sets of stock DNAs were obtained from each extraction. The first sample, obtained by "spooling" a DNA precipitate, and a less pure sample obtained by centrifugation of the residual sample.

Samples of DNA produced in this way were mixed in known concentrations to provide comparative results.

The DNA obtained in this way was then analysed using touchdown PCR with SEQ ID Nos. 4 and 5 shown above as forward and reverse primers respectively. This technique involves the gradual reduction of annealing temperature every few cycles during the amplification procedure. This has the effect of reducing primer-binding to target

sequences in the template DNA which do not have a perfect complimentary sequence, thereby increasing the reaction specificity. In this case, the following conditions were employed:

Programme No	No of cycles	Linked to programme No	Segments
8	1	1	3 min @ 94°C
1	2	2	30s @ 94°C
			1 min @ 68°C
			30s @ 72°C
2	2	3	30s @ 94°C
			1 min @ 66°C
			30s @ 72°C
3	2	4	30s @ 94°C
			1 min @ 64°C
			30s @ 72°C
4	2	5	30s @ 94°C
			1 min @ 62°C
			30s @ 72°C
5	30	6	30s @ 94°C
			1 min @ 60°C
			30s @ 72°C
6	1	7	5 min @ 72°C
7	1	0	99 h @ 4°C

The following PCR reagents were used:

Water	15µl
Cresol red	5µl (10 mg/ml dissolved in 60% sucrose)
10x PCR Buffer (see below)	2.5µl (Mg^{2+} conc. 15mM)
DNTP's	1µl (2.5mM)
Forward primer	0.25µl (20µM)
Reverse primer	0.25µl (20µM)
NP40	0.25µl (5%)
Tween-20	0.25µl (5%)
1µl DNA	
0.1µl Taq	

10x PCR buffer -	100mM Tris.HCl pH 8.3
	500mM KCl
	15 mM $MgCl^2$
	0.1% (m/v) gelatin

All oligonucleotides used as primers were synthesised on Millipore Expedite oligonucleotide synthesiser using Expedite™ chemistry. They were purified by first uncrimping the column and pouring glass beads into screw-capped Eppendorf tube. 300µl ammonia was added and the reaction tube incubated at 55°C for 30-60 minutes. The tube was then placed tube on ice for 5 minutes and centrifuged at 12000 rpm for 2 minutes. The supernatant was added to a new screw-cap tube, and 30µl 3M ammonium acetate, and 3 volumes cold ethanol added. The mixture was placed in -70°C freezer for 30-60 minutes and then spun at 12000 rpm 15 minutes at 4°C. The supernatant was aspirated and the resultant pellet washed briefly with cold (-20°C) 80% ethanol. After allowing the pellet to dry, it was resuspended in 200µl ddH_2O and the concentration measured.

The results are shown in Figure 5. The PCR showed increasing band strength on the gel as the level of contamination with hexaploid wheat increased.

In similar experiments, the primers SEQ Id Nos 4 and 6 and reverse primers 5 and 7 used in more or less any combination, appear to allow significantly enhanced D-genome specific amplification, than any of the other primer combinations, including the primers used in the McNeil et al. paper.

Example 3

PCR using Multiplex PCR

During investigation of the Dgas44 sequence for suitably PCR primers, it was found that certain primers were none specific and would detect both the A & B as well as the D genome. One such pair of primers comprises SEQ ID No 8 and 9 as follows:

AGCAACTCCCCCTTAGCGGAGCAC (SEQ ID NO 8)

CAGCTCCGAGTCAGAGGTAACC (SEQ ID NO 9)

(SEQ Id no 8 is similar to that disclosed in McNeil et al., supra but without the 3'T). These primers are based upon the sequences found from base Nos 5-28 and 855-876 respectively in theDgas44 sequence (see Figure 1)

Since the amplification product using primers of SEQ Id Nos 8 and 9 is significantly larger than the within-domain D-genome specific products produced in Example 2, it was thought that this would provide a useful internal control for successful DNA amplification. Example 2 was therefore repeated using the crop DNA as well as the pasta DNA but using a combination of two groups of primers comprising SEQ Id No 4, SEQ Id No5, SEQ Id No 8 and SEQ Id No 9. The results are shown in Figure 6.

It is clear that the addition of the two non-specific primers provides a useful internal standard or control against which the level of adulteration may be assessed.

Example 4

Quantification using short PCR product

Using PCR conditions substantially as described above and following the scheme shown in Figure 4 and described in Forster et al. supra, short PCR products have been generated using the following steps:

1) As forward primer:

CTTCTGACGGGTCAGGGGCAC (SEQ ID NO 4)

and as the reverse primer

CTGAATGCCCCTGCGGCTTAAG (SEQ ID NO 5);

2. As forward primer

CTTCTGACGGGTCAGGGGCAC (SEQ ID NO 4)

and as the reverse primer in place of SEQ ID No 5

GCGGCTTAAGCTGTAGATTTCCGGCT (SEQ ID NO 17)

or GCGGCTTAAGTTCATAAGCCGCCACC (SEQ ID NO 18)

based upon regions 2050-2065, and 2077-2092 as well as 2129-2138 of the Dgas 44 sequence as shown in Figure 1.

PCR in this instance was performed using a touchdown 58-54°C protocol using the product derived in step 1) as DNA template.

3. As forward primer:

CTTCTGACGGGTCAGGGGCAC (SEQ ID NO 4)

and as the reverse primer:

CTGAATGCCCCTGCGGCTTAAG (SEQ ID NO 5).

PCR in this instance was performed using a touchdown 58-54°C protocol using the product derived in step 2) as DNA template.

The touchdown PCR protocol can be summarised as follows:

Programme No	No of cycles	Linked to programme No	Segments
8	1	1	3 min @ 94°C
1	2	2	30s @ 94°C
			1 min @ 58°C
			30s @ 72°C
2	2	3	30s @ 94°C
			1 min @ 56°C
			30s @ 72°C
3	2	4	30s @ 94°C
			1 min @ 54°C
			30s @ 72°C
4	1	7	5 min @ 72°C
7	1	0	99 h @ 4°C

The existence of the desired short-PCR product was confirmed on a 4% agarose gel.

When these products were used as a competitor in PCR reactions using pasta DNA obtained as described above in Example 2, it was found that increasing the amount of short-PCR product, reduces the amount of the full-length PCR product. Since the amount of short-PCR product added to the reactions was known, the original amount of contaminating Dgas44 present in the pasta samples can be calculated by a comparison of the signals.

The short-PCR product has been cloned into the PGEM-T cloning vector (Promega, Madison, WI, USA).

Example 5

Cultivar screening

To assess the applicability of the protocol to determine the presence of the D-Genome in a uniform manner a wide range of *T. durum* and *T. aestivum* cultivars were gathered from around the world. *T. durum* cultivars were collected (55 accredited samples and 3 unaccredited) representing the major cultivars in common commercial usage and the growing areas around the world. In addition, a number of accessions from the John Innes germplasm collection were added to the screening procedure. The origins of these rare cultivars were diverse, yet they represent the genetic diversity sometimes utilised within breeding programmes. They were therefore included to strengthen the robustness of the protocol particularly with respect to new cultivars that may be bred as a result of crosses with these varieties. The large number of cultivars allowed an assessment of the uniformity of detection of the D-Genome. It is known that the Dgas44 sequence is repeated many times within the D-Genome, however, the exact number of repeats and the question of whether or not the number of repeats is constant between cultivars is unknown.

In an attempt to cover the major sources of *T. aestivum* contamination a large collection (66) of cultivars have been gathered. Most of the cultivars are in current commercial cultivation however where these were not available then slightly older cultivars have

been substituted. The collection represented the most common wheat growing areas of the world and hence covers the most probable sources of contamination/adulteration.

In addition, a selection of 38 samples of the related *Triticum* species and suspected donors of the various genomes were included in the screen in order to evaluate the unlikely possibility that other genomes may interfere with the specific detection of the D-genome. These samples were examined 'blind'. .

In addition to the species and cultivars described above a limited number of *Tritordeum* lines (*H. chilense* (a wild barley) crossed with either *T. turgidum* or *T. aestivum*) were examined due to their potential use within the food industry and the possibility of cross contamination of the Spanish durum crop.

DNA was extracted from all of the cultivars shown by the method described in Example 2 above for pasta.

Following the analysis of the extracted DNA for the presence of the D- genome by the PCR method as described above and using SEQ ID Nos 4 and 5 as primers, the results were as follows:-

1. DNA samples extracted from *T. aestivum* cultivars all produced the characteristic amplification product indicative of the presence of the D-genome when subjected to PCR.

2. All the DNA's extracted from accredited *T. durum* cultivars failed to produce the amplified product indicative of the presence of the D-Genome.

Three *T. durum* accessions from the John Innes Centre germplasm collection that had been included as more diverse examples of the species did however produce an amplified product. This indicated that the D-genome was present in these materials. Further analysis of these accessions by Acid-PAGE showed that in every case that the

putative pure *T. durum* cultivar that had been included in the germplasm collection as a *T. durum* cultivar was either a *T. aestivum* or a mixture of the two species.

3. The analyses of the nine amphiploid lines showed that the specific amplified product (indicative of the presence of a D-genome) was obtained only from the three primary octaploid lines (*H. chilense* X *T. aestivum*), HT4, HT73 and HT 116. The primary and secondary hexaploids (*H.chilese* X *T. turgidum)* all failed to produce a specific amplified product.

4. The results obtained from the analyses of other *Triticum* and related species (lines 1-37, appendix 13) proved to be extremely interesting both in terms of the specificity and strength of the detection of the D-Genome, with the majority of the D-Genomes being detected. There were however, a few non-conforming results. One of the most surprising results involved *T. palecolchicum* which gave a weakly positive result despite being tetraploid and having only the A and B Genomes. This species however is unusual in that it has only ever been found growing with hexaploid wheat, it is therefore assumed to be a regression product and hence the reason for the positive result. It was also noticeable that while the species carrying the D-Genome produce a reasonably strongly amplified product the amphiploid lines and substitution lines produce weakly amplified products. Langdon (1A) 1D and *Ae. vavilovi & Ae. juvenalis* gave negative or weakly amplification. In the case of Langdon (1A) 1D it is possible that the 1D chromosome carries very few copies of the Dgas44 sequence and therefore not detectable using our assay.

Overall, the results of this analysis are in very good agreement with the expectation on the basis of the genomic constitution of the various lines or species.

Example 6

Detection using Southern Blotting

DNA extracted from pasta as described above, was digested to completion with the following combinations of endonucleases were used :-*Taq*I, *Eco*RI/*Bam*H1, *Eco*RV/*Sst*I, *Bam* HI /*Bgl* II. DNA extracted from *T. durum* plant leaves as described above in Example 2 was also prepared in this way to act as positive controls.

A probe was prepared by PCR from the Dgas44 sequence using the following as primer sequences:

AGCAACTCCCCCTTAGCGGAGCAC (SEQ ID NO 8)

GGTGACTCCATGCAGTCAAATCTG (SEQ ID NO 20)

SEQ ID No 12 comprises a forward primer based upon base 5 to 29 of the Dgas44 sequence and SEQ ID no 20 comprises a reverse primer based upon bases 408 to 431 of the Dgas 44 sequence.

The thus produced probe was radiolabelled using inserts with ^{32}P by the method of Feinberg and Vogelstein, (1983). Anal. Biochem. 132, 13-16.

Hybridisation was performed with a probe made by PCR from plasmids containing the Dgas44 sequence using the method of Southern, E.M. (1975). *Detection of specific sequences among DNA fragments separated by gel electrophoresis.* J.Mol.Biol. 98. 503-517.

The positive controls were very radioactive compared to the pasta dilutions, giving in some instances overexposure of the film in certain areas. The pasta samples also gave a signal but this was weaker.

Example 7

Dot blotting

A dot blot assay was performed. DNA extracted from pure *T. durum* was used as a positive control as well as DNA extracted from the 56° pasta prepared as described above.

A nitrocellulose membrane was cut to 9 x 12cm and washed in a buffer comprising 175.2g sodium chloride, and 88.2g sodium citrate per litre, adjusted to pH 7.0 with NaOH (SSC) for 10-15 min. and drained well.

The membrane was placed on a sealing gasket which was fixed into dot blotting apparatus which was attached to a vacuum. 100µl 20x SSC was loaded to each well and then drained with a full vacuum for approx. 5 min. Subsequently DNA at concentrations of 1, 2, 5 and 10µg/well (in 60µl 0.4 M NaOH solution) was loaded to each well. After gentle vacuuming for 20 min until wells look empty and dry.

The membrane was removed and rinsed in 2x SSC for 15 min. After blotting dry, the membrane was subjected to hybridisation as described above in Example 5 and the results are shown in Figure 7.

This shows that increasing contamination makes a difference which is detectable on hybridisation and these differences are visible to the naked eye. This may be quantified using densitometry.

Example 8

PCR using different primers

Primers of SEQ ID Nos 8 and 20 were used in a touchdown PCR reaction at conditions 68-58°C (Don R.H et al., (1991) Nucleic Acids Research **19**, 4008) to amplify DNA prepared as described in Example 2 above. The reagents used in a

standard 50µl reaction per tube was as follows:

Water	30µl (or 40µl if no cresol red used)
Cresol red	10µl (10mg/ml dissolved in 60% sucrose)
10x PCR buffer	5µl (Mg^{2+}, 15mM)
DNTP's	2µl (2.5mM)
Forward primer	0.5µl (20µM)
Reverse primer	0.5µl (20µM)
NP40	0.5µl (5%)
Tween-20	0.5µl (5%)
1µl DNA	(20ng/ul)

For single copy sequences 0.2µl Taq (5U/µl)

(For repeated sequences 0.1µl Taq was used)

Although the results were of variable quality and there were problems with very feint and diffuse bands (probably due to the high copy number of this sequence in the genome giving rise to a large amount of PCR products, not all of precisely the same length and possibly also some degree of length polymorphism among the genomic copies of this sequence). These problems can be minimised to a degree by appropriate modification of the PCR conditions.

With these primers, a temperature of 60°C was found to be optimum and increasing the buffer/Mg^{2+} concentration to 2.0mM was suitable. The concentration of Taq polymerase which gave sharper bands was 0.25U, with a primer concentration of x0.5. A representative result is shown in Figure 8.

This indicates that contamination at levels as low as 1% may be detected with this method.

Example 8

Quantitative PCR using TAQMAN™ Probe

PCR reactions were carried out using primers of SEQ ID Nos 4 and 5 as the forward and reverse primers respectively and in addition a TAQMAN ™ probe of SEQ ID No 10.

$^{5'}$TTG GGA GGC ATG GTG AAA GTT GGT GAT$^{3'}$ (SEQ ID NO 10)

Other conditions were as follows:

1. **Cycler Conditions**

STEP 1---------	1 Cycle	50°C	2 min
STEP 2---------	1 Cycle	95°C	10min
STEP 3---------	45 Cycles	95°C 60°C	15sec 1min

(All temperature changes at 1°C / Sec)

2. PCR REACTION MIXTURE CONSTITUENTS (25µl total per tube)
(final tube concentrations given)

10X Buffer (a PE- Applied Biosystems proprietry mix of unknown composition)	
Magnesium Chloride	5mM
dATP	200µM
dGTP	200µM
dCTP	200µM
dUTP	400µM
Forward Primer	300nM
Reverse Primer	300nM
Taqman Probe	200nM
AmpliTaq DNA polymerase	0.02U/µl
Uracil N-glycosidase	0.01U/µl
water	

Inclusion of this probe allowed quantification of the *T. aestivum* content of the sample. Development of the fluorescent signal from the TAQMAN ™ probe was monitored in the conventional manner. Examples of the results are illustrated in Figures 11 to 13.

These figures show the fluorescence detected in the PCR reaction as a function of the PCR cycle number. The fluorescence detected is in proportion to the amount of specific target sequence amplified in the reaction. The software used allows the determination of the cycle threshold value (Ct) which is the cycle or part of it when the detected fluorescence differs from the 0% standard by 6 standard deviations.

They show the decrease in Ct as the adulteration level increases; i.e. the number of target sequences present in these samples is increasing, which in turn least to a fluorescent signal of equal intensity at an earlier cycle time.

The Ct value was then plotted against the known adulteration level within the standards and a series of calibration curves plotted (Figure 14). By combining this data. a calibration curve against which pastas of unknown heating history can be measured was derived (Figure 15).

Example 9

Amplication of the PSR 128 intron using Touchdown PCR

Samples prepared as described in Example 1 were subjected to a touchdown PCR reaction as follows:

Forward primer	$^{5'}$ATG GCT GGC TTC TAT TTC ATG$^{3'}$	(SEQ ID No. 12)
Reverse primer	$^{5'}$CAC CTA CTC CTC CAC ACT TTG$^{3'}$	(SEQ ID No. 13)

STEP		
1	94°C	3min
2	94°C	30sec
3	62°C	1min
4	72°C	1min
5	94°C	30sec
6	60°C	1min
7	72°C	1min
(steps 5-7 a total of 3 times)		
8	94°C	30sec
9	58°C	1min
10	72°C	1min
(steps 8-10 a total of 3 times)		
11	94°C	30sec
12	56°C	1min
13	72°C	1min
(steps 11-13 a total of 3 times)		
14	94°C	30sec
15	54°C	1min
16	72°C	1min
(steps 11-13 a total of 3 times)		
17	94°C	30sec
18	54°C	1min
19	72°C	1min
(steps 17-19 a total of 3 times)		
20	94°C	30sec
21	54°C	1min
22	72°C	1min
(steps 20-22 a total of 3 times)		
23	94°C	30sec
24	52°C	1min

25 72°C 1min
(steps 23-25 a total of 24 times)

26 10°C 20h

(all temperature changes were at 0.5°C / sec)

REACTION MIXTURE (for 50µl reactions)

	µl
water	12
Cresol red	10
10X PCR Buffer (Boehringer)	5 (15mM Mg^{2+})
dNTP's	5 (2mM)
Forward primer	5 (2µM)
Reverse primer	5 (2µM)
NP40	2.5 (1%)
Tween-20	2.5 (1%)
DNA	3 (20µg/µl)
Taq (Boehringer Manheim)	0.2 (5U/µl)

Amplication of the target sequence was achieved.

Example 10

A TAQMAN™ Assay for PSR128

Using similar conditions to those described in Example 9 above, samples prepared as described in Example 1 were subjected to quantitative PCR using the following reagents.

Forward primer $^{5'}$AAG GAG CTC GCC AAC GG$^{3'}$ (SEQ ID No 14)

Reverse Primer $^{5'}$AAC CGA GGG TCC AGA AGA GAC G$^{3'}$ (SEQ ID No 15)

TAQMAN™ probe:

$^{5'}$GCT AGA TGG CTG GCT TCT ATT TCA TGA TCC A$^{3'}$ (SEQ ID No 19)

Calibration curves similar to those described in Example 8 were prepared (Figures 16 and 17). Reliable and accurate quantification of the amount of *T. aestivum* was possible using this method.

5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(D) STATE: Perthshire
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(A) NAME: Gale, Michael Dennis
(B) STREET: 9 Mount Pleasant
(C) CITY: Norwich
(E) COUNTRY: Great Britain
(F) POSTAL CODE (ZIP): NR2 2DG

(ii) TITLE OF INVENTION: Detection Means

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2296 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCAGCAAC TCCCCCTTAG CGGAGCACTT GGCCAACACG GTCGGTCTCC CCGGCTCAAT 60

GTACTCCGTC CGGGTGATTA CCACGTCCGG GTCGTCTGCT GGTGGAGCTG TAGCGGAGGC 120

TCCGGGTTAA ATGAAGCTTC GGTCGTTGCC TTAGTAGTTG GGGCGGTGGC TTCAGGTGCC 180

GTGTCCATTG GAGTGGCGGC TTCGGGGGCG GAGGCAGCTG GCGGTTCTTG GACTGTCACC 240

GCCGGCTCAG GCAAATCCGG CACTCCGGCC GACTTGGTCT TCTTCACCCT CTTGGTGAGC 300

TTTGCTTGGG CACTGAAAGA ACAACAAAAG TTAGTACAAA GCATATGAGT AAAGATCAGA 360

ACAACATGAG ATGATTGTTG TTACCCGGGC GCGGTCTTGA AAGCCGGCAG ATTTGACTGC 420

ATGGAGTCAC CCGAAGAGGG GGAGGTCTCC TGATAATTGG AGTCAGAAGA GTTGAGTGGC 480

TGACGGATAA CACCCGCCAA CGGTGAAAAG GGTACAAGGG GAAATAACCT CAGTTCGGCG 540

TTTCCTTGTT GCGTCGGGTA ACCCGGACGG AGAGGTCGGT GTCCTTGTTG GTCCGGGTGT 600

GACGCCGGCT TTCGTGAACC TGTCGCTTCA AAGAGAACAA TTGAGGGTCT TGATGAGCTA 660

AAGGATGTGA AAAGCTTACT TTCCGGGTTA CCCTTCCAGA CTTTCAGCTG TGGCAAGGGC 720

TCCGAGTCGG AGGAAAGTGA CATTACCTCT TCAACCACCG GGTTACTGGC GCCGGTGTCA 780

TCCTGTCAAT GAGCAAGTGT TGATAAGGCG GACAACAACA AACAACAAAT GCAGCAAGAA 840

TTTAAAGGCT TAAGGGTTAC CTCTGACTCG GAGCTGTNGC TTAATGTGCA TCAGCTCCGA 900

AGCAGTAGGT CTGCTTCCGT TTTTGCGAGG GGCGGCTTTC TTGGCGGCTT TCTTTGCCTT 960

CCTGGCTTTC TTGGCCGCCT CATGGTCATA CTTGACCCGC CAGAACTTAT CATCAGCCTG 1020

GCAAAAATAC AATGAGGAAT TATTAAAACA GGTTTAGATG AAGGTTATAT GCAGACGAAG 1080

ATAAACAGTT AAAGTCAGCG GCTTACGCTG GGGCTGGATT GGTCTTGCAG AAGGGGACTA 1140

GCCCGGTCCT CCACAGTCTG GCGAGGGCTC TCGTTTAAAA GAGCCTTGGT CTCCTCCTCT 1200

GCAACGTCTT CTGGAAGATC ATTGCGCTGT GCCTCTTGGG GTCATCCTTT CACCCGGTGT 1260

ACTCACACAT TAAGCCGGGG ACGGCGGCTC AATGGGATCA CCCGCCATGA GATCCAGACC 1320

CGGACCAGGT CGATTCCATT CAGACCGTTG CCAGGAGGGC CTTGATTTTG TTGATAGTAG 1380

GAAGCAGAGG CTGGTGCTCC GCTGGAGTCA GCTTGTCCGA CAGTGGGTGA GTCGGCTCTA 1440

GACGTGTTGG GCGAAAGCCG GGCAGCGGGC TTTCATCAGC CGGGGACGTG TCTTGGCAGT 1500

AGAACCAAGT CATGTTCCAG TCCTTGGGGT GGCTCGGCGG CTCTACGTAA GGGAAAAGAC 1560

AGTCCCTACG CCGCTGGATG GAAATGCCAC CTAACTCCAG ACTCGGCCCG TTGGCGCACT 1620

CATTCTGGCG GTTTAAGTAA AACAGCTCTC TGAAGAGCAG CAAACTAGGC TCTTCTCCAA 1680

GGTACACTTC GCAAAAGACT TGGAAATTGC AGATGTTGGA TACGGAGTTG GGTCCTATAT 1740

CTTGAGGCCG CAAGTCAAAG AAGTTGAGCA CGTCCCTGAA AAACTTTGAG CCGGGTGGTG 1800

CGAAGCCCCG GCTCATATGA TCTGCGAAAA TCACTACCTC CCCGTCCTTT GGCTGTGGTC 1860

TCCCTTCTGA CGGGTCAGGG GCACGGTAGG ACATGATTTC CTTCTTAGGC AAATATCCGG 1920

ACTTCACAAA ATTGGCTAGG GTCTCATTGG TGACGTTGAC CTCATCCAGT TGCAAGTGAT 1980

GGGAGCCTTG GGAGGCATGG TGAAAGTTGG TGATCTATGA CAAAAAGGAA AATGTCCGGG 2040

TTAATTATAA GCCGGAAATC TACAGGTCAA AACTAAGGTG GCGGCTTATG AAGGGGACTA 2100

ATGATATATA CTCAGGTACA GTGGGTTACT TAAGCCGCAG GGGCATTCAG AATAAGTTGA 2160

TGATCTACGG CCTTATCACA GTTAAGCCGG AGGAATCTAC AGCGCGTGGT TTCTTTAAAC 2220

CGGAATTATA AGCCGCCAAG ATTCAATGGT TGCAGTTTTT TACTAAGTGT GGTAAAACAG 2280

GTTTCACATA CTGCAG 2296

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 419 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTCCTATATC TTGAGGCCGC AAGTCAAAGA AGTTGAGCAC GTCCCTGAAA AACTTTGAGC 60

CGGGTGGTGC GAAGCCCCGG CTCATATGAT CTGCGAAAAT CACTACCTCC CCGTCCTTTG 120

GCTGTGGTCT CCCTTCTGAC GGGTCAGGGG CACGGTAGGA CATGATTTCC TTCTTAGGCA 180

AATATCCGGA CTTCACAAAA TTGGCTAGGG TCTCATTGGT GACGTTGACC TCATCCAGTT 240

GCAAGTGATG GGAGCCTTGG GAGGCATGGT GAAAGTTGGT GATCTATGAC AAAAAGGAAA 300

ATGTCCGGGT TAATTATAAG CCGGAAATCT ACAGGTCAAA ACTAAGGTGG CGGCTTATGA 360

AGGGGACTAA TGATATATAC TCAGGTACAG TGGGTTACTT AAGCCGCAGG GGCATTCAG 419

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 287 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTTCTGACGG GTCAGGGGCA CGGTAGGACA TGATTTCCTT CTTAGGCAAA TATCCGGACT 60

TCACAAAATT GGCTAGGGTC TCATTGGTGA CGTTGACCTC ATCCAGTTGC AAGTGATGGG 120

AGCCTTGGGA GGCATGGTGA AAGTTGGTGA TCTATGACAA AAAGGAAAAT GTCCGGGTTA 180

ATTATAAGCC GGAAATCTAC AGGTCAAAAC TAAGGTGGCG GCTTATGAAG GGGACTAATG 240

ATATATACTC AGGTACAGTG GGTTACTTAA GCCGCAGGGG CATTCAG 287

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTCTGACGG GTCAGGGGCA C 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGAATGCCC CTGCGGCTTA AG 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTCCTATATC TTGAGGCCGC AAG 23

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AACCCACTGT ACCTGAGTAT ATATC 25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGCAACTCCC CCTTAGCGGA GCAC 24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAGCTCCGAG TCAGAGGTAA CC 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTGGGAGGCA TGGTGAAAGT TGGTGAT 27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTCGAGAAC AAGGAGAAGG AGCTCGCCAA CGGTACCTCC ATAGTCTCTC TTCTTCTTCT 60

TCTTTTTTCA AATATCCAGC TAGATGGCTG GCTTCTATTT CATGATCCAT CGTCTCTTCT 120

GGACCCTCGG TTCCTAATCG GACGGCTCTT GTTAATTTTT GGATTTTTTT TTCATTCATT 180

CATTCAGGTA GACTGGCGAT 200

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGGCTGGCT TCTATTTCAT G 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CACCTACTCC TCCACACTTT G 21

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGGAGCTCG CCAACGG 17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AACCGAGGGT CCAGAAGAGA CG 22

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCTAGATGGC TGGCTTCTAT TTCATGATCC A 31

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGGCTTAAG CTGTAGATTT CCGGCT 26

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCGGCTTAAG TTCATAAGCC GCCACC 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 549 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCTCGAGAAC AAGGAGAAGG AGCTCGCCAA CGGTACCTCC ATAGTCTCTC TTCTTCTTCT 60

TCTTTTTTCA AATATCCAGC TAGATGGCTG GCTTCTATTT CATGATCCAT CGTCTCTTCT 120

GGACCCTCGG TTCCTAATCG GACGGCTCTT GTTAATTTTT GGATTTTTTT TTCATTCATT 180

CATTCAGGTA GACTGGCGAT GCTGGCGTTC CCGGGGTTCC TGGTGCAGCA CAACGTGACC 240

GGCAAGGGCC CGTTCGAGAA CCTGCAGCAG CACCTGGCCG ACCCATGGCA CACCACCATC 300

ATCCAGACCA TCTCCGGCCA GTAAATCCGT CGATTTCACC GACCGGGGAG GTTTCAGGTG 360

GTCTGAATTG TTATTTTGGG GTACTGAGGA TGTACAAAGT GTGGAGGAGT AGGTGGACAG 420

TGCAAAAATG TGTTGTAATC TTAAAGCTCC GGTGGTGAAA CTTTCTTCTT GTGGATTTCG 480

CCATGGCTTC CGATGGAGGC TGGACTTGTA ATATCACCCA TGCGTGTTAT TCTAAACATG 540

GCAAAACTA 549

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTGACTCCA TGCAGTCAAA TCTG 24

Claims

1. A method for detecting the presence of wheat having a D-genome in a processed food product, which method comprises extracting DNA from said product and detecting the presence of a nucleotide sequence which is characteristic of the D-genome.

2. A method according to claim 1 wherein said food product comprises flour, semolina, or pasta products.

3. A method according to claim 1 or claim 2 wherein the said nucleotide sequence is amplified prior to or during detection.

4. A method according to claim 3 wherein the amplification is carried out using the polymerase chain reaction (PCR).

5. A method according to any one of the preceding claims wherein the nucleotide sequence which is detected is present as a single copy in the D-genome.

6. A method according to claim 3 wherein the nucleotide sequence which is detected is a D-genome specific derivative of the PSR 128 sequence shown in Figure 9.

7. A method according to claim 6 wherein a derivative of the PSR128 sequence is amplified using a pair of primers selected from either

$^{5'}$ATG GCT GGC TTC TAT TTC ATG$^{3'}$ (SEQ ID No. 12) and

$^{5'}$CAC CTA CTC CTC CAC ACT TTG$^{3'}$ (SEQ ID No. 13); or

$^{5'}$AAG GAG CTC GCC AAC GG$^{3'}$ (SEQ ID No 14)

$^{5'}$AAC CGA GGG TCC AGA AGA GAC G$^{3'}$ (SEQ ID No 15).

8. A method according to claim 7 wherein the pair of primers used are

5' AAG GAG CTC GCC AAC GG 3' (SEQ ID No 14) and

5' AAC CGA GGG TCC AGA AGA GAC G 3' (SEQ ID No 15).

9. A method according to any one of claims 1 to 4 wherein the nucleotide sequence detected comprises or is a D-genome specific derivative of the Dgas44.

10. A method according to claim 9 wherein a characterising portion of the sequence shown in Figure 2 is amplified and detected.

11. A method according to claim 10 wherein the sequence amplified comprises the sequence of Figure 3.

12. A method according to claim 10 or claim 11 wherein the amplification is carried out using forward primers selected from either:

CTTCTGACGGGTCAGGGGCAC (SEQ ID NO 4)

or GTCCTATATCTTGAGGCCGCAAG (SEQ ID NO 6)

and reverse primers selected from either

CTGAATGCCCCTGCGGCTTAAG (SEQ ID NO 5)

or AACCCACTGTACCTGAGTATATATC (SEQ ID NO 7).

13. A method according to claim 12 wherein the forward primer comprises

CTTCTGACGGGTCAGGGGCAC (SEQ ID NO 4)

and the reverse primer comprises

CTGAATGCCCCTGCGGCTTAAG (SEQ ID NO 5).

14. A method according to any one of claims 1 to 3 wherein the nucleotide sequence is detected by Southern or Dot blotting using a radiolabelled probe derived from the said characteristic sequence.

15. A method according to any one of the preceding claims wherein the amount of said wheat present in the product is quantified.

16. A method according to claim 15 wherein a second nucleotide sequence of said DNA is also amplified, said second sequence being found on the A and B and D genomes, the second nucleotide sequence is also detected and used as an internal standard.

17. A method according to claim 16 wherein said second nucleotide sequence is derived from the Dgas44 sequence.

18. A method according to claim 17 wherein said second nucleotide sequence comprises all or a substantial non-D-genome specific portion of a region of the Dgas44 sequence defined by base 5 to 876 of sequence of Figure 1.

19. A method according to claim 15 wherein the quantification is effected by amplifying said target sequence by the using a pair of primers in the presence of a known concentration of a further sequence which competes with the said sequence for a primer, but which is distinguishable from said sequence during detection. and comparing signals produced by said further sequence and said target sequence.

20. A method according to claim 15 wherein the quantification is effected using fluorescence based analysis.

21. A method according to claim 20 wherein the amplification reaction is effected in the presence of a probe which includes a quencher moiety and a fluorescence reporter, said probe being arranged to be selectively cleaved during the amplification reaction so as to produce a flurorescent signal.

22. A method according to claim 21 wherein the amplification of a region of the PSR128 intron is effected using as primers

$^{5'}$AAG GAG CTC GCC AAC GG$^{3'}$ (SEQ ID No 14) and

$^{5'}$AAC CGA GGG TCC AGA AGA GAC G$^{3'}$ (SEQ ID No 15) ;

and the probe comprises a sequence

$^{5'}$GCT AGA TGG CTG GCT TCT ATT TCA TGA TCC A$^{3'}$ (SEQ ID No 16)

23. A method according to claim 21 wherein the amplification of a region of Dgas44 is carried out using the primers defined in claim 13, and the probe comprises a sequence

$^{5'}$TTG GGA GGC ATG GTG AAA GTT GGT GAT$^{3'}$ (SEQ ID NO 10).

24. A nucleotide sequence which comprises or hybridises with all or part of the D-genome nucleotide sequence shown in Figure 8 (SEQ ID No 11).

25. A nucleotide probe or primer which is specific for the nucleotide sequence of claim 24.

26. A pair of amplification primers which comprise either

CTTCTGACGGGTCAGGGGCAC (SEQ ID NO 4)

or GTCCTATATCTTGAGGCCGCAAG (SEQ ID NO 6)

and either CTGAATGCCCCTGCGGCTTAAG (SEQ ID NO 5)

or AACCCACTGTACCTGAGTATATATC (SEQ ID NO 7)

27. A pair of primers according to claim 26 which comprise SEQ ID NOS 4 and 5.

28. A pair of amplification primers which are selected from either

$^{5'}$ATG GCT GGC TTC TAT TTC ATG$^{3'}$ (SEQ ID No. 12) and

$^{5'}$CAC CTA CTC CTC CAC ACT TTG$^{3'}$ (SEQ ID No. 13); or

$^{5'}$AAG GAG CTC GCC AAC GG$^{3'}$ (SEQ ID No 14) and

$^{5'}$AAC CGA GGG TCC AGA AGA GAC G$^{3'}$ (SEQ ID No 15).

29. A kit for detecting the presence of wheat having a D-genome in a processed food product, said kit comprises either a labelled probe which hybridises with a sequence which is specific for the D-genome, or means for amplifying such a sequence.

30. A kit according to claim 29 wherein the said sequence which is specific for the D-genome is or is derived from the Dgas44 sequence or from the PSR128 intron sequence.

31. A kit according to claim 29 or claim 30 which comprises a pair of primers according to any one of claims 26 to 28.

32. A kit according to any one of claims 27 to 30 which further comprises other reagents required to effect the polymerase chain reaction.

32. A kit according to any one of claims 27 to 31 which further comprises means for extracting DNA from said processed food product.

33. A method for identifying the presence of a D-genome in a crop sample, which method comprises extracting DNA from said sample, and detecting the presence of a nucleotide sequence which comprises or hybridises with all or part of the nucleotide sequence shown in Figure 2.

34. A method according to any one of claims 29 to 33 wherein the detection is effected by amplifying said sequence using PCR.

Abstract

A method for detecting the presence of wheat having a D-genome in for example a processed food product is described. The method comprises extracting DNA from said product and detecting the presence of a nucleotide sequence which is characteristic of the D-genome and in particular is derived from the Dgas44 sequence or from the PSR128 intron. The sequence is suitably detected using PCR.

A particular sequence within each of the Dgas44 and the single copy PSR128 intron has been identified as being a suitable target for detection.

The method is useful in detecting contamination of flour, semolina, or pasta products which are made from *T. durum* by bread wheat.

Reagents useful in the method as well as kits for conducting the method are also described and claimed.

Fig.1. 1/4

```
   1  CTGCAGCAAC TCCCCCTTAG CGGAGCACTT GGCCAACACG GTCGGTCTCC
  51  CCGGCTCAAT GTACTCCGTC CGGGTGATTA CCACGTCCGG GTCGTCTGCT
 101  GGTGGAGCTG TAGCGGAGGC TCCGGGTTAA ATGAAGCTTC GGTCGTTGCC
 151  TTAGTAGTTG GGGCGGTGGC TTCAGGTGCC GTGTCCATTG GAGTGGCGGC
 201  TTCGGGGGCG GAGGCAGCTG GCGGTTCTTG GACTGTCACC GCCGGCTCAG
 251  GCAAATCCGG CACTCCGGCC GACTTGGTCT TCTTCACCCT CTTGGTGAGC
 301  TTTGCTTGGG CACTGAAAGA ACAACAAAAG TTAGTACAAA GCATATGAGT
 351  AAAGATCAGA ACAACATGAG ATGATTGTTG TTACCCGGGC GCGGTCTTGA
 401  AAGCCGGCAG ATTTGACTGC ATGGAGTCAC CCGAAGAGGG GGAGGTCTCC
 451  TGATAATTGG AGTCAGAAGA GTTGAGTGGC TGACGGATAA CACCCGCCAA
 501  CGGTGAAAAG GGTACAAGGG GAAATAACCT CAGTTCGGCG TTTCCTTGTT
 551  GCGTCGGGTA ACCCGGACGG AGAGGTCGGT GTCCTTGTTG GTCCGGGTGT
 601  GACGCCGGCT TTCGTGAACC TGTCGCTTCA AAGAGAACAA TTGAGGGTCT
 651  TGATGAGCTA AAGGATGTGA AAAGCTTACT TTCCGGGTTA CCCTTCCAGA
```

Fig.1. 2/4

```
 701  CTTTCAGCTG TGGCAAGGGC TCCGAGTCGG AGGAAAGTGA CATTACCTCT
 751  TCAACCACCG GGTTACTGGC GCCGGTGTCA TCCTGTCAAT GAGCAAGTGT
 801  TGATAAGGCG GACAACAACA AACAACAAAT GCAGCAAGAA TTTAAAGGCT
 851  TAAGGGTTAC CTCTGACTCG GAGCTGTNGC TTAATGTGCA TCAGCTCCGA
 901  AGCAGTAGGT CTGCTTCCGT TTTTGCGAGG GGCGGCTTTC TTGGCGGCTT
 951  TCTTTGCCTT CCTGGCTTTC TTGGCCGCCT CATGGTCATA CTTGACCCGC
1001  CAGAACTTAT CATCAGCCTG GCAAAAATAC AATGAGGAAT TATTAAAACA
1051  GGTTTAGATG AAGGTTATAT GCAGACGAAG ATAAACAGTT AAAGTCAGCG
1101  GCTTACGCTG GGGCTGGATT GGTCTTGCAG AAGGGGACTA GCCCGGTCCT
1151  CCACAGTCTG GCGAGGGCTC TCGTTTAAAA GAGCCTTGGT CTCCTCCTCT
1201  GCAACGTCTT CTGGAAGATC ATTGCGCTGT GCCTCTTGGG GTCATCCTTT
1251  CACCCGGTGT ACTCACACAT TAAGCCGGGG ACGGCGGCTC AATGGGATCA
1301  CCCGCCATGA GATCCAGACC CGGACCAGGT CGATTCCATT CAGACCGTTG
1351  CCAGGAGGGC CTTGATTTTG TTGATAGTAG GAAGCAGAGG CTGGTGCTCC
```

Fig.1. 3/4

```
1401  GCTGGAGTCA GCTTGTCCGA CAGTGGGTGA GTCGGCTCTA GACGTGTTGG
1451  GCGAAAGCCG GGCAGCGGGC TTTCATCAGC CGGGGACGTG TCTTGGCAGT
1501  AGAACCAAGT CATGTTCCAG TCCTTGGGGT GGCTCGGCGG CTCTACGTAA
1551  GGGAAAAGAC AGTCCCTACG CCGCTGGATG GAAATGCCAC CTAACTCCAG
1601  ACTCGGCCCG TTGGCGCACT CATTCTGGCG GTTTAAGTAA AACAGCTCTC
1651  TGAAGAGCAG CAAACTAGGC TCTTCTCCAA GGTACACTTC GCAAAAGACT
1701  TGGAAATTGC AGATGTTGGA TACGGAGTTG GGTCCTATAT CTTGAGGCCG
1751  CAAGTCAAAG AAGTTGAGCA CGTCCCTGAA AAACTTTGAG CCGGGTGGTG
1801  CGAAGCCCCG GCTCATATGA TCTGCGAAAA TCACTACCTC CCCGTCCTTT
1851  GGCTGTGGTC TCCCTTCTGA CGGGTCAGGG GCACGGTAGG ACATGATTTC
1901  CTTCTTAGGC AAATATCCGG ACTTCACAAA ATTGGCTAGG GTCTCATTGG
```

Fig.1. 4/4

```
1951  TGACGTTGAC CTCATCCAGT TGCAAGTGAT GGGAGCCTTG GGAGGCATGG
                                                        <
2001  TGAAAGTTGG TGATCTATGA CAAAAAGGAA AATGTCCGGG TTAATTATAA
2051  GCCGGAAATC TACAGGTCAA AACTAAGGTG GCGGCTTATG AAGGGGACTA
2101  ATGATATATA CTCAGGTACA GTGGGTTACT TAAGCCGCAG GGGCATTCAG
2151  AATAAGTTGA TGATCTACGG CCTTATCACA GTTAAGCCGG AGGAATCTAC
2201  AGCGCGTGGT TTCTTTAAAC CGGAATTATA AGCCGCCAAG ATTCAATGGT
2251  TGCAGTTTTT TACTAAGTGT GGTAAAACAG GTTTCACATA CTGCAG
```

Fig.2.

GTCCTATAT CTTGAGGCCG CAAGTCAAAG AAGTTGAGCA CGTCCCTGAA

AAACTTTGAG CCGGGTGGTG CGAAGCCCCG GCTCATATGA TCTGCGAAAA

TCACTACCTC CCCGTCCTTT GGCTGTGGTC TCCCTTCTGA CGGGTCAGGG

GCACGGTAGG ACATGATTTC CTTCTTAGGC AAATATCCGG ACTTCACAAA

ATTGGCTAGG GTCTCATTGG TGACGTTGAC CTCATCCAGT TGCAAGTGAT

GGGAGCCTTG GGAGGCATGG TGAAAGTTGG TGATCTATGA CAAAAAGGAA

AATGTCCGGG TTAATTATAA GCCGGAAATC TACAGGTCAA AACTAAGGTG

GCGGCTTATG AAGGGGACTA ATGATATATA CTCAGGTACA GTGGGTTACT

TAAGCCGCAG GGGCATTCAG

Fig.3.

CTTCTGA CGGGTCAGGG GCACGGTAGG ACATGATTTC CTTCTTAGGC

AAATATCCGG ACTTCACAAA ATTGGCTAGG GTCTCATTGG TGACGTTGAC

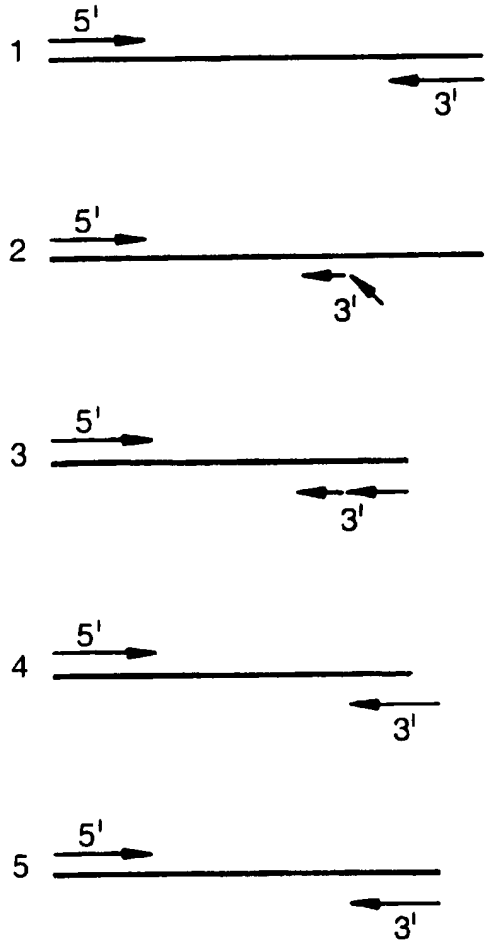
CTCATCCAGT TGCAAGTGAT GGGAGCCTTG GGAGGCATGG TGAAAGTTGG

TGATCTATGA CAAAAAGGAA AATGTCCGGG TTAATTATAA GCCGGAAATC

TACAGGTCAA AACTAAGGTG GCGGCTTATG AAGGGGACTA ATGATATATA

CTCAGGTACA GTGGGTTACT TAAGCCGCAG GGGCATTCAG

Fig.4.



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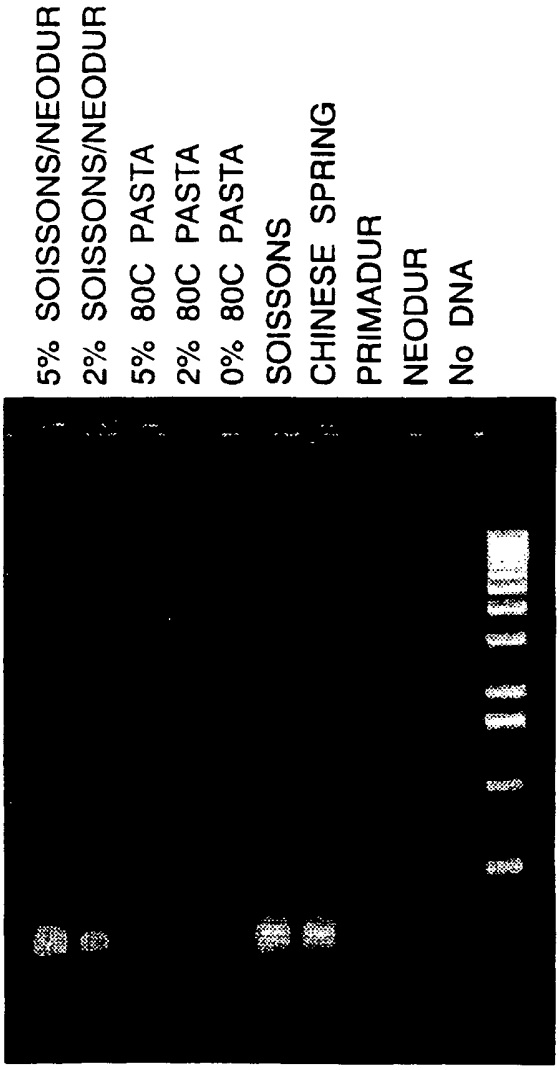
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NEODUR
No DNA

Fig.6.

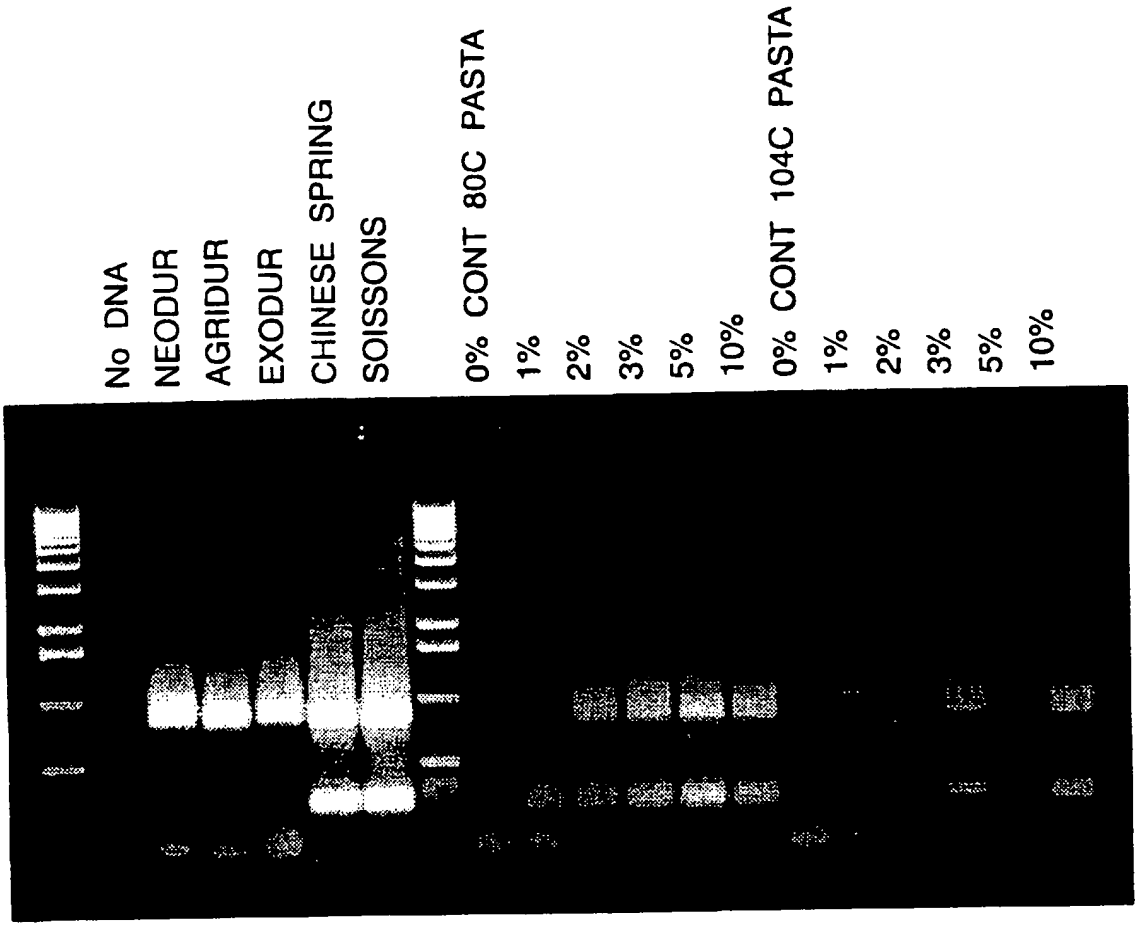
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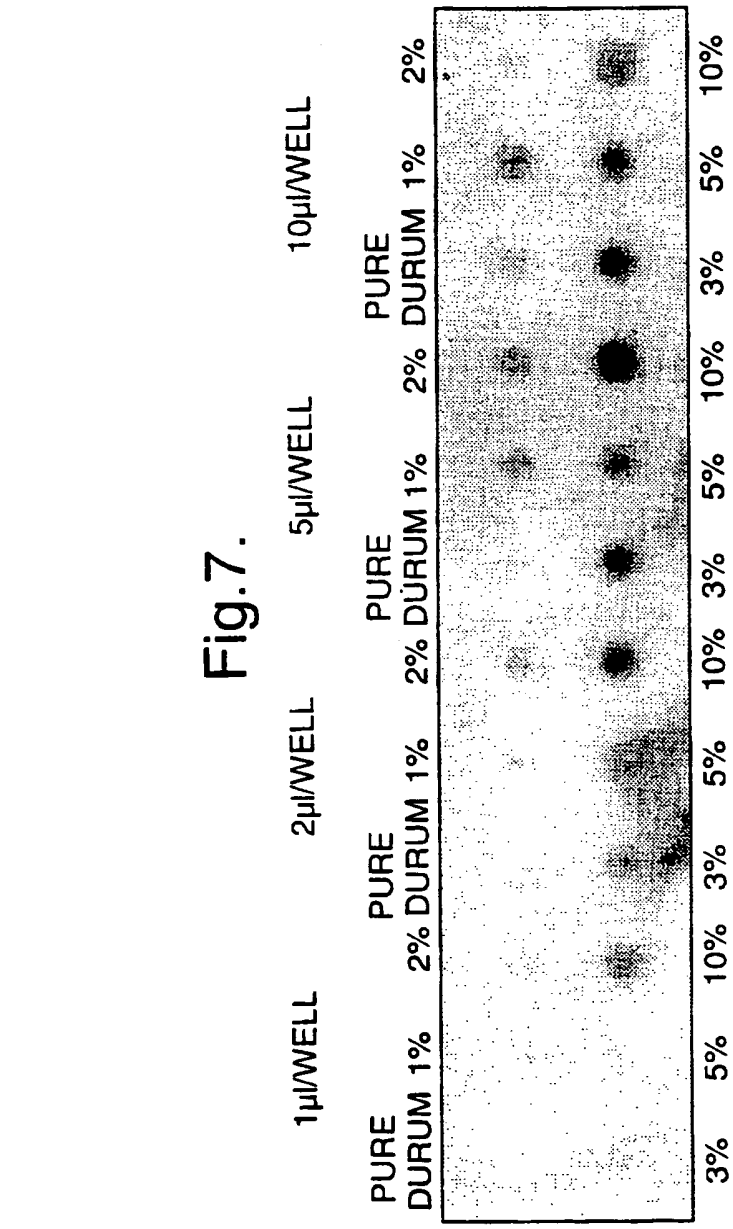
Fig.7.



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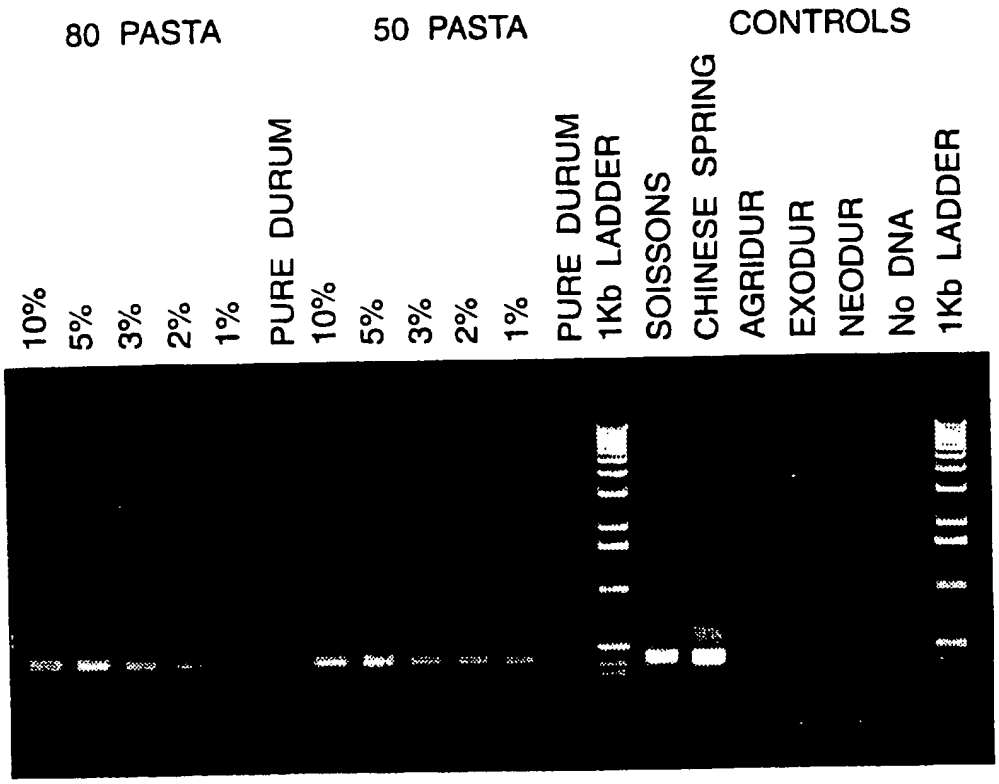
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SOISSONS
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Fig.9.

1	CCTCGAGAAC	AAGGAGAAGG	AGCTCGCCAA	CGGTACCTCC	ATAGTCTCTC
	TTCTTCTTCT	TCTTTTTTCA	AATATCCAGC	TAGATGGCTG	GCTTCTATTT
101	CATGATCCAT	CGTCTCTTCT	GGACCCTCGG	TTCCTAATCG	GACGGCTCTT
	GTTAATTTTT	GGATTTTTTT	TTCATTCATT	CATTCAGGTA	GACTGGCGAT
201	GCTGGCGTTC	CCGGGGTTCC	TGGTGCAGCA	CAACGTGACC	GGCAAGGGCC
	CGTTCGAGAA	CCTGCAGCAG	CACCTGGCCG	ACCCATGGCA	CACCACCATC
301	ATCCAGACCA	TCTCCGGCCA	GTAAATCCGT	CGATTTCACC	GACCGGGGAG
	GTTTCAGGTG	GTCTGAATTG	TTATTTTGGG	GTACTGAGGA	TGTACAAAGT GTTTCA
401	GTGGAGGAGT CACCTCCTCA	AGGTGGACAG TCCAC	TGCAAAAATG	TGTTGTAATC	TT.AAAGCTC
	CGGTGGTGAA	ACTTTCTTCT	TGTGGATTTC	GCCATGGCTT	CCGATGGAGG
501	CTGGACTTGT	AATATCACCC	ATGCGTGTTA	TTCTAAACAT	GGCAAAACTA

Fig.10.

1 CCTCGAGAAC AAGGAGAAGG AGCTCGCCAA CGGTACCTCC ATAGTCTCTC

TTCTTCTTCT TCTTTTTTCA AATATCCAGC TAGATGGCTG GCTTCTATTT

101 CATGATCCAT CGTCTCTTCT GGACCCTCGG TTCCTAATCG GACGGCTCTT
GCAGAGAAGA CCTGGGAGCC AA

GTTAATTTTT GGATTTTTTT TTCATTCATT CATTCAGGTA GACTGGCGAT

FORWARD PRIMER 5' AAG GAG CTC GCC AAC GG 3'

REVERSE PRIMER 5' AAC CGA GGG TCC AGA AGA GAC G 3'

TAQMAN PROBE 5' GCT AGA TGG CTG GCT TCT ATT TCA TGA TCC A 3'

Fig.11.

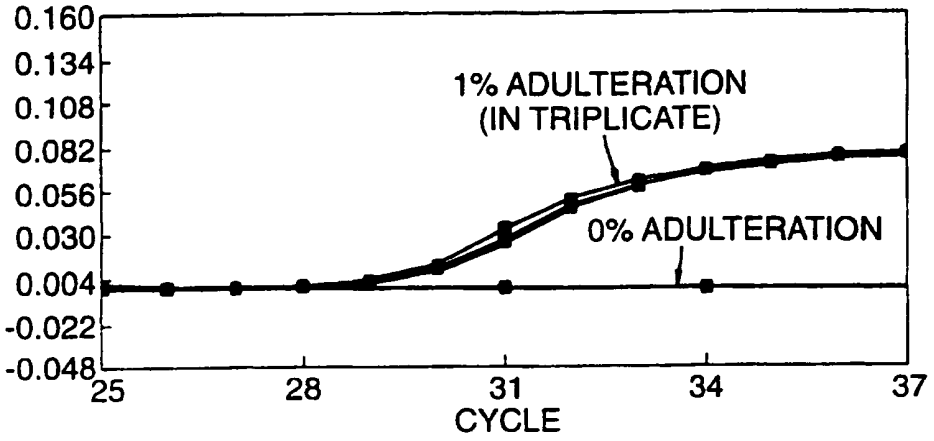

Fig.12.

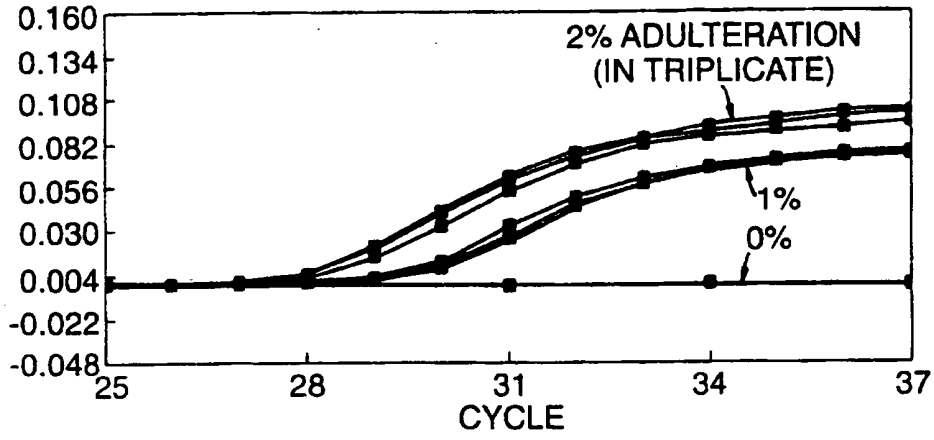

Fig.13.

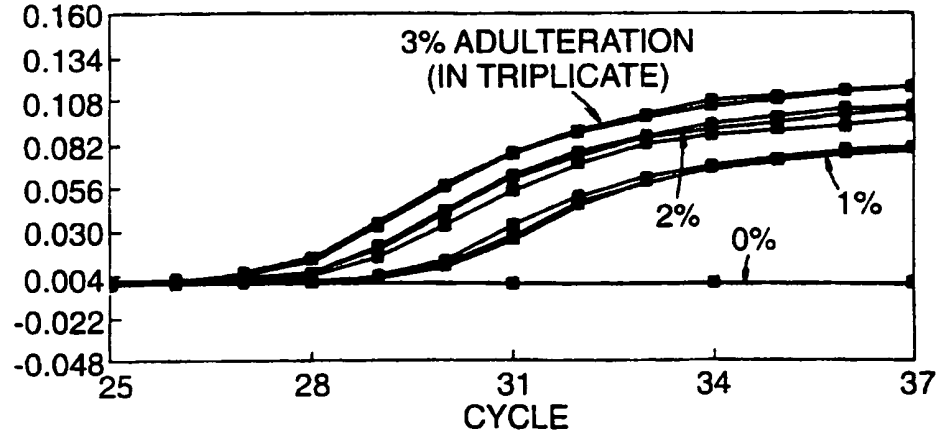

Fig.14.

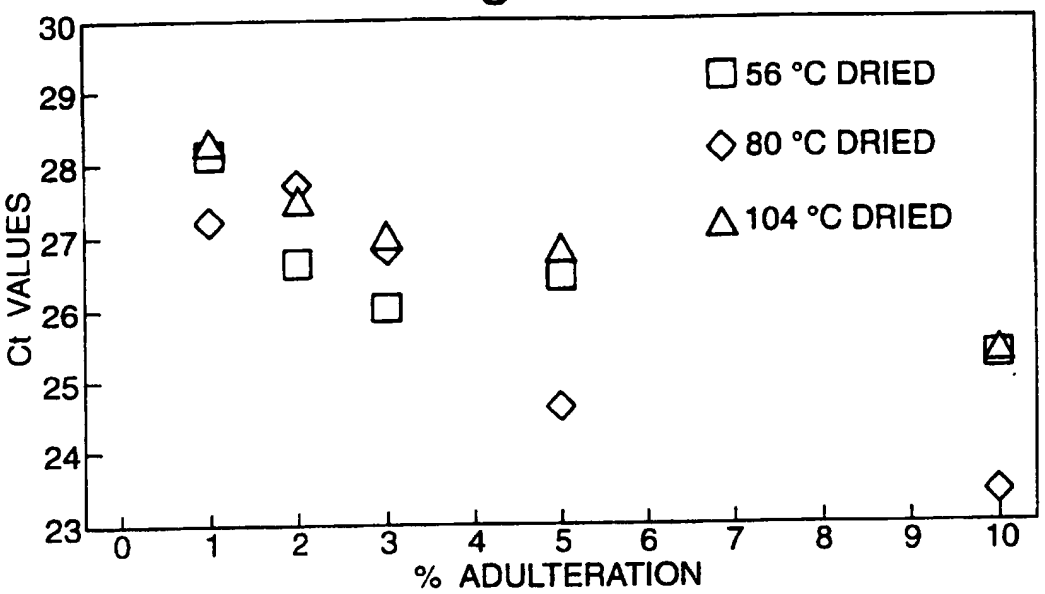
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Fig.15.

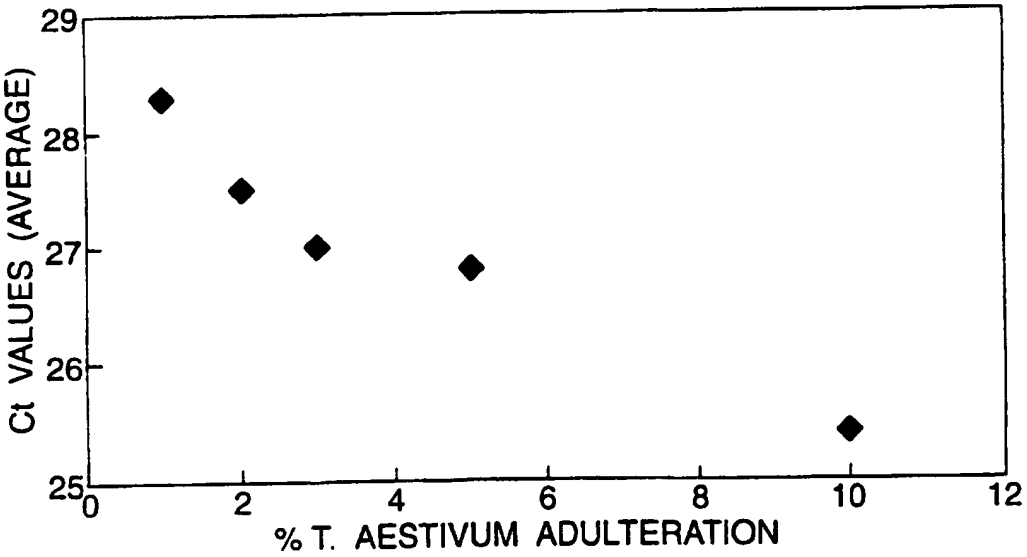
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Fig.16.

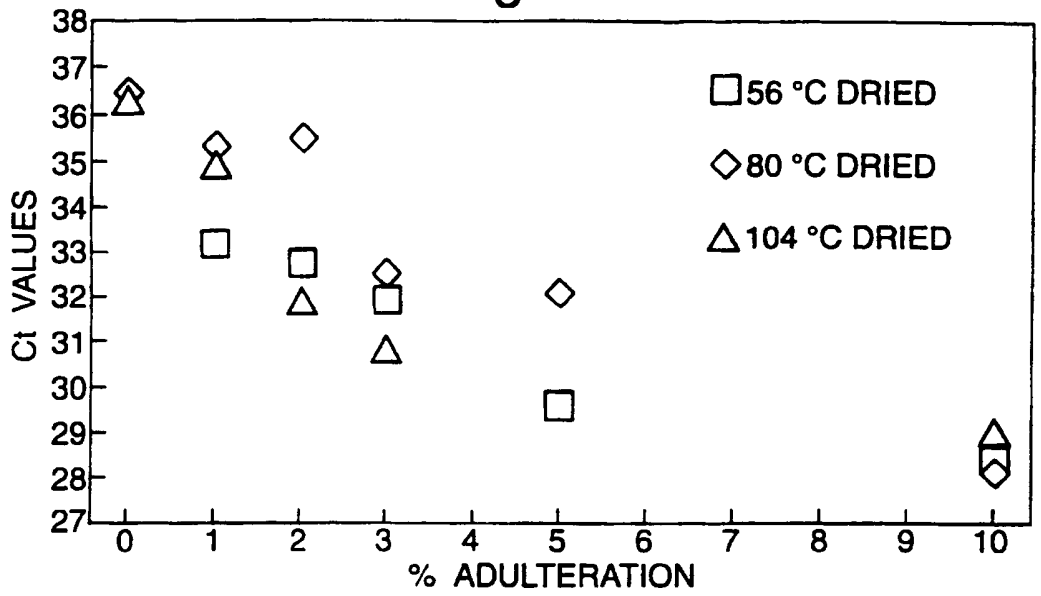
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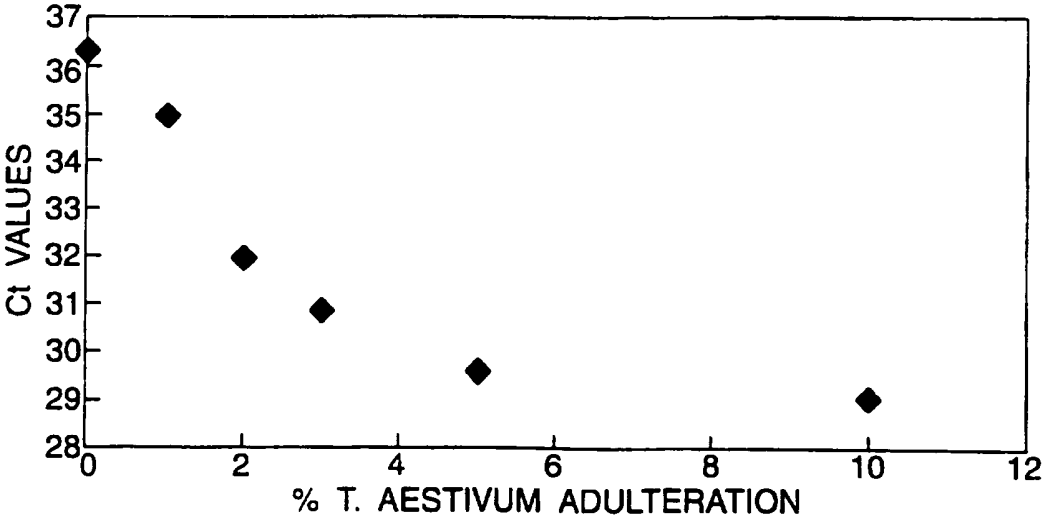
Fig.17.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INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/GB 97/01988

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BUSCH W. ET AL.,: "Repeated DNA sequences isolated by microdissection. I. Karyotyping of barley (Hordeum vulgare L.)" GENOME, vol. 38, - 6 December 1995 pages 1082-1090, XP002046287 see abstract and results, esp. p.1084,2.para.; p.1089, 2. para. --- -/--	1.5,14, 33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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2

Date of the actual completion of theinternational search	Date of mailing of the international search report
13 November 1997	28/11/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/GB 97/01988

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCNEIL D. ET AL.,: "Amplification of DNA sequences in wheat and its relatives: the Dgas44 and R350 families of repetitive sequences" GENOME, vol. 37, - April 1994 pages 320-327, XP002046288 cited in the application see abstract and materials & methods	1,3-5, 9-11, 14-19, 29-32
A	RAYBURN A. L. & GILL B. S.: "Molecular analysis of the D-genome of the triticea" THEOR. APPL. GENET., vol. 73, - 1987 pages 385-388, XP002046850 see the whole document	1
X,P	DE 195 25 284 A (INST PFLANZENGENETIK UND KULTU) 2 January 1997 see the whole document	1,3-5, 29,32-34

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter...ional Application No
PCT/GB 97/01988

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19525284 A	02-01-97	WO 9701567 A	16-01-97